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Biotransformation of Reactive Black HEBL into 3 nitroso-3-azabicyclo (3.2.2) nonane by an acclimated mixed culture

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Abstract: An isolated and acclimated mixture culture exhibited a versatile capability to decolorize an azo dye; Reactive Black HEBL (RBHEBL) under static condition with a higher average decolorization rate. The mixed culture gave best decolorization performance with partial mineralization of 70.2% COD reduction. The UV-vis spectrophotometric, FT-IR, HPLC and GC-MS analyses confirmed the decolorization was due to degradation by the mixed culture. The biotransformation of RBHEBL resulted in the formation of 3-nitroso-3-azabicyclo (3.2.2) nonane by the enzyme lignin peroxidise (LiP) induced by the mixed culture. The phytotoxicity studies using *Phaseolus mungo* and *Triticum aestivum* revealed the reduced toxic nature of the biotransformed product when compared with the initial RBHEBL.
Keywords: Biotransformation, Reactive Black HEBL, 3-nitroso-3-azabicyclo (3.2.2) nonane, mixed culture.

Introduction

The textile dyeing and finishing industry use wide variety of dyestuffs due to the rapid changes in the customer's demands. Huge amount of dyes in textile sectors are continuously being exhausted in wastewater streams due to their poor adsorbability to the fibre[1-2]. It is estimated that about 10-15% of the total production of colorants is lost during their synthesis and dyeing processes[3-4]. Color present in dye effluent gives a straightforward indication of water being polluted, and discharge of this highly colored effluent can damage directly the receiving water. Poor analytical methods and the complexity of manufacturing and retailing are also contributing to the environmental damage caused by these coloring compounds. In aquatic systems, the dyes undergo various reactions and the alterations to their chemical structures can result in the formation of new xenobiotic compounds, which may be more or less toxic than the parental compounds[5]. Chemical and physical methods for treatment of dye wastewater are not widely applied to textile industries because of exorbitant costs and disposal problems. Insufficient treatment of wastes of the dyestuff industries leads to dye contamination of the environment such as soil and natural water bodies[6].

The new environment-friendly regulations concerning textile products ban marketing of textiles dyed with azo dyes capable of reductively splitting into carcinogenic aromatic amines and they also ban the

discharge of colored waste. These laws, coupled with the problems resulting from physicochemical treatment of azo dyes, have generated interest in the wider use of biodegradation which is the predominant natural mechanism in soil[7].

Biodegradation is an environmental friendly and cost competitive alternative to chemical decomposition processes[8-9]. Microorganisms have been isolated, selected, mutated and genetically engineered for effective bioremediation[10-11] including the ability to degrade recalcitrant pollutants, and achieve enhanced rates of degradation of target compounds. The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms.

Over the past decades, many microorganisms are capable of degrading azo dyes, including bacteria, fungi, yeast, actinomycetes and algae[12-19]. The low pH requirement for an optimum activity of the enzymes and the long hydraulic retention time for complete decolorization are the disadvantages of using fungi. In addition, they may inhibit the growth of other useful microorganisms. Thus, large-scale applications of fungal decolorization have been limited. Most of the metabolic studies have been limited to bacterial genera; however, since azo dyes are considerably recalcitrant[20-21].

The present work focuses on the decolorization ability of the isolated mixed culture on to RBHEBL under varying process parameters. The intermediates formed after dye decolorization was analyzed using UV-vis spectrophotometric, FT-IR, HPLC and GC-MS analysis. The extent of detoxification was assessed using phytotoxicity assays.

Materials and Methods

Dyestuff, chemicals and instruments

The textile dye Reactive Black HEBL was obtained from Bi-Ki Dyes and Chemicals Pvt. Ltd., India. Double distilled water was used for preparing dye stock solutions throughout the study and the stock solution was stored in the dark at room temperature. The nutrient broth and agar were purchased from Himedia (Mumbai, India). All chemicals were of the highest purity and of an analytical grade.

Instrumental analysis

The pH measurements were carried out with a microprocessor based pH meter model number HI 98107, Hanna Equipments Private Limited, Mumbai, India. The decolorization was estimated using absorbance recorded on Shimadzu UV-1800 spectrophotometer model number (Tokyo, Japan). The chemical oxygen demand (COD) measurements were done using the COD reflux apparatus Spectra Lab, 2015D (Mumbai, India). The biomass was sonicated to disrupt the cell wall using sonics-vibracell ultrasonic processor. The FT-IR spectra were measured in Perkin Elmer 237B Infrared spectrometer. The HPLC analyses were carried on Shimadzu, Japan, equipped with a UV-vis detector in C_{18} column (symmetry, 4.6x250 mm). The GC-MS analyses were carried on Agilent 5975 C Shimadzu, Japan equipped with a HP-5 capillary column (30 m x 0.25 mm x 0.25 μm film thickness).

Isolation and screening of RBHEBL degrading microorganism

The microorganisms present in the soil samples from the effluent disposal site of a textile-dyeing industry located in Trippur, India were enriched in the nutrient broth NB medium containing (g/L): beef extract, 3; peptone, 10; NaCl, pH 6.6 and incubated at 37˚C for 24 h in a rotary orbital shaker at 150 rpm. The culture possessing the ability to decolorize RBHEBL (100 mg/L) was inoculated 1% (v/v) in NB medium incubated at 37˚C for 24 h. An aliquot of 1 mL of cell suspension was transferred into fresh RBHEBL containing NB was done to screen whether the culture possess the decolorizing ability. The screening procedure in liquid medium was continued until decolorization of the RBHEBL containing broth.

Acclimatization of RBHEBL degrading mixed culture

Acclimatization is the ability of microbial strain to adapt or adjust to the changing environment. Acclimation usually involves the use of alternative metabolic pathways by the microbial strain which is not disrupted or least disrupted to a lesser degree by the presence of RBHEBL. When the acclimatized culture reached the exponential phase, 1.0 mL of the culture medium was transferred to the next culture medium. The acclimatization studies were carried out at 37˚C under static condition and the pure acclimated culture was maintained as glycerol stock and preserved at -20˚C.

Decolorization experiment

The 24 h grown cells were incubated with RBHEBL at a concentration of 100 (mg/L) and incubated at 37˚C under static condition. An aliquot (3 mL) of the culture media was withdrawn at the different time intervals. Aliquot was centrifuged at 10,000 rpm for 15 min to separate cell mass. Supernatant was used to determine the decolorization by measuring the change in absorbance of culture supernatants at the maximum absorption wavelength (λ_{max}) of corresponding dyes. All decolorization experiments were performed in triplicates. Abiotic (without microorganisms) controls were always included. Decolorization was quantitatively analyzed using UV-vis spectrophotometer and during UV-vis spectral analysis; changes in absorption spectrum in the decolorized medium were recorded in comparison with the results from the control runs. The percentage of decolorization was calculated as follows,

$$\%Decolorization = \frac{Initial\ absorbance\ -Observed\ absorbance}{Initial\ absorbance} \times 100 \quad (1)$$

The average decolorization rate, ADR_{RBHEBL}(μg/min) was calculated as follows,

$$ADR_{RBHEBL}(\mu g/\min) = \frac{C \times \%D \times 1000}{100 \times t} \quad (2)$$

where C is the initial concentration of dye (mg/L) and %D is the dye decolorization (%) after time t (min).

Degree of mineralization through chemical oxygen demand measurement

The COD of the solution *ie.,* before and after decolorization was measured to determine the degree of mineralization of RBHEBL (initially with 100 mg/L), with the mixed culture. The samples were centrifuged at 10,000 rpm for 10 min and the supernatant was used for COD analysis. To determine the extent of mineralization, the reduction in COD, the percentage COD reduction after decolorization was evaluated as follows:

$$(\%)\ COD\ reduction = \frac{Initial\ COD_{(0h)} - Observed\ COD_{(t)}}{Initial\ COD_{(0h)}} \times 100 \quad (3)$$

where $COD_{(0\ h)}$ and $COD_{(t)}$ are the initial and final COD value after a particular reaction time (t), respectively.

Extraction and characterization of metabolites formed after decolorization

The decolorization was quantitatively analyzed using a UV-vis spectrophotometer, whereas the biodegradation was monitored by FT-IR spectroscopy, HPLC and GC-MS. The culture media was centrifuged after decolorization after respective incubation time at 7000 rpm for 20 min. The metabolites presents in culture supernatant were extracted using equal volume of ethyl acetate, dried over anhydrous sodium sulphite (Na_2SO_3) and evaporated to dryness in rotary vacuum evaporator. The biotransformed products were characterized by FT-IR and compared with control. The FT-IR analysis was done in the mid-IR region of 400-4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder and the analyses were carried out. The HPLC analysis was carried out by dissolving the rotary vacuum evaporated metabolites in HPLC grade methanol. The chromatogram of RBHEBL and the metabolites were scanned at 519 and 666 nm by the UV-vis detector. The mobile phase was methanol with a flow rate of 1.0 mL/min with 10 min run time and an aliquot (10 μL) of the sample was manually injected into the injector port. The HPLC grade methanol dissolved metabolites were used for GC-MS analysis by initially setting the column at 70˚C for 5 min and then programmed to reach 150˚C at a rate of 5˚C/min, while maintaining the operating temperature of injector and detector at 260˚C with an ionization voltage of 70 eV. The helium (He) was used as carrier gas with a flow rate 1.0 mL/min for 30 min run time. The unknown spectrum, Apex was integrated using ChemStation Integrator and the compounds were identified on the basis of mass spectra and using the NIST library.

Phytotoxicity assessment

The phytotoxicity of the dye and their extracted metabolites was carried out at room temperature tested in the concentration range of 500 ppm. Tests were carried out on the commonly used seeds, *Triticum aestivum* (20 seeds) and *Phaseolus mungo* (10 seeds) placed on two layers of filter papers by watering separately 5 mL of control dye in petriplates, maintaining light and temperature in a controlled environment. Control set was

carried out using distilled water at the same time. Percentage germination (% GP), plumule (shoot) radicle (root) and chlorophyll content of seedlings were reported after 7 days.

Results and Discussion

Screening and acclimation of the mixed culture

It has been observed that microorganisms indigenous to the contamination are efficient in degrading xenobiotic or recalcitrant compounds. The acclimated mixed culture was developed by the culture enrichment method and it was observed that the mixed cells represent an inexpensive and promising tool for the removal of various dyes from textile effluent as they possess a synergistic effect and attack the dye molecules at different positions. The decolorizing capacity of microorganisms in the mixed culture can be tested by examining its potential to degrade RBHEBL and the decolorization was due to microbial action not because of the pH variation and this was confirmed by monitoring the change in pH level and was observed that the pH was in the range 6.5–7.0.

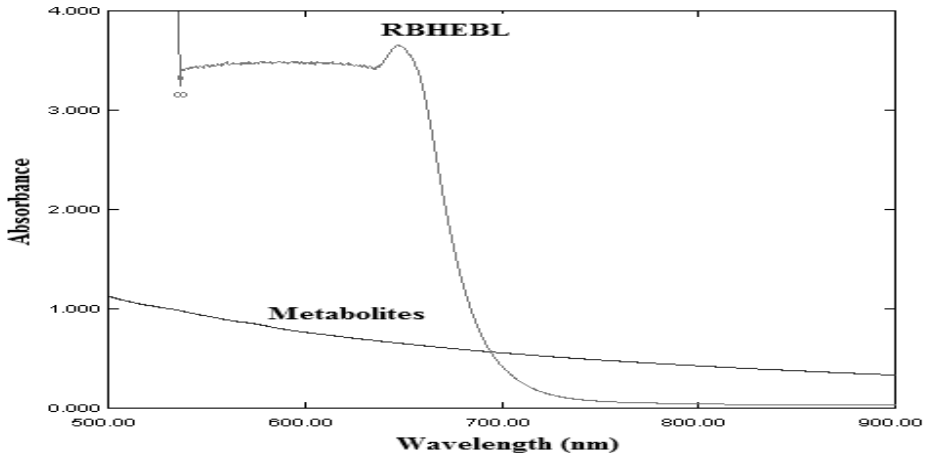
Reduction in COD level of RBHEBL

The degree of mineralization of the RBHEBL, when treated with the mixed culture was measured after the decolorization, at respective time periods of incubation. The percent COD reduction was varying for the different RBHEBL concentration. The maximum percent COD reduction of 77.87% was observed for 100 (mg/L) RBHEBL, indicating partial mineralization. It is evident that the individual strains in the mixed culture consumed the dye and their reaction intermediates as the carbon source and they exhibited a synergistic effect to partially reduce the COD level considerably. Thus, from the economical as well as environmental point of view, the mixed culture could be a good alternative to physico-chemical methods for dye decolorization and mineralization.

Analysis of biotransformation

Azo dyes can be cleaved symmetrically or asymmetrically, with an active site available for an enzyme to excite the molecule and the asymmetric cleavage by LiP between the nitrogen of the azo group and the carbon of the aromatic ring resulted in the formation of 3-nitroso-3-Azabicyclo [3.2.2] nonane. In order to disclose the possible mechanism of dye decolorization, the products of the biotransformation of RBHEBL was analyzed by UV-vis spectroscopy whereas, biodegradation was monitored by FT-IR, HPLC and GC-MS analysis. The UV-vis absorbance (from 300 to 900 nm) of RBHEBL incubated in the decolorization medium was monitored by a UV-vis spectrophotometer to examine the decolorization, by the mixed culture. The major visible light absorbance peak disappeared completely, in the UV-vis spectra (Figure- 1) confirming that the decolorization was completely due to the metabolism mediated by the consortium.

Figure 1: UV-vis spectra of RBHEBL and biodegraded product by the consortium

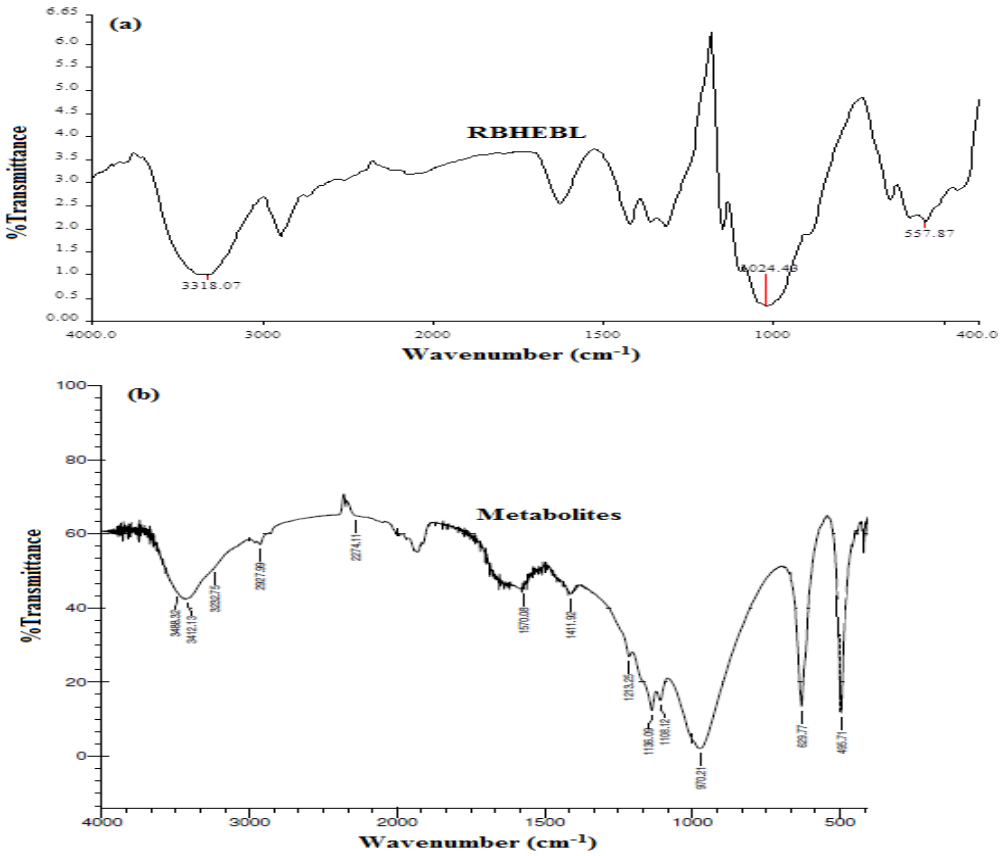


To disclose the possible mechanism of RBHEBL degradation, the product of biotransformation was analyzed using UV- vis spectrophotometry. The λ_{max} was observed at 597 nm, whereas the degradation

products for 100 (mg/L) was 306 nm. The UV-vis absorption peak disappeared completely, thus showing that biodegradation has occurred.

The nature of the degradation products obtained was confirmed by the FT-IR analysis and the variations in the fingerprint region (1500 to 500 cm^{-1}) of the FT-IR spectroscopy of the control RBHEBL and the extracted metabolites indicate biodegradation by the consortium (Figure- 2).

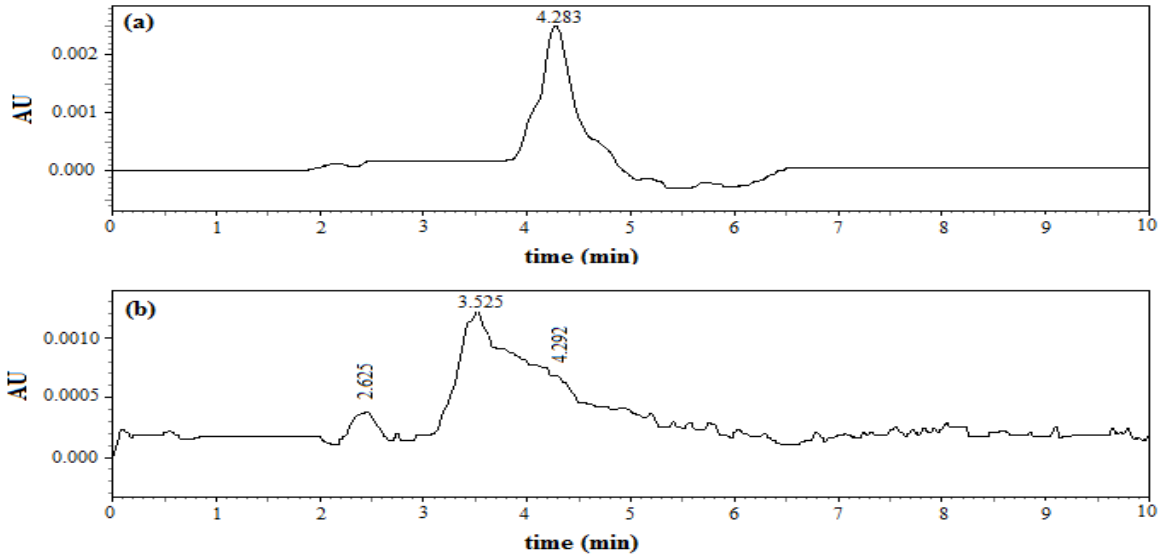
Figure 2: FT-IR spectra of (a) control RBHEBL and (b) extracted metabolites



The FT-IR spectra of control RBHEBL showed peaks in fingerprint region (1500 to 500 cm^{-1}) for the alkyl ethers which are supported by a peak at 1024 cm^{-1} for the C-O stretching and for the stretching of primary alcohol. The peak at 557 cm^{-1} indicates the C-S stretching vibration and also indicates the presence of halogenated group. The peak at 3318 cm^{-1} gives the perception for O-H stretching of phenols for the RBHEBL structure. The FT-IR spectra of degraded metabolites formed after RBHEBL degradation by the mixed culture showed peaks in fingerprint region (1500 to 500 cm^{-1}) for the alkyl ethers which are supported by a peak at 1136 cm^{-1} for the C-O stretching. The peak at 629 cm^{-1} confers the presence of OH bending of alcohol at out of plane bend and also indicates the C-C bending vibration. The peak at 1108 cm^{-1} describes the bending vibrations for the C-O esters and two distinct bands for the C-H vibration. The peak 1136 cm^{-1} indicates the presence of sulfone group and the peak at 1213 cm^{-1} for C-N stretching of aliphatic amines. The strong vibrations at 1411 cm^{-1} for the CH alkanes and primary amines (N-H stretching vibrations) at 1570 cm^{-1} indicates the breakdown of complex dye into simpler products. The absence of peak at 1367 cm-1 confirms the absence of azoic groups in the degraded products. The ammonium ions at 2927 cm^{-1} and the supporting peaks at 3412 and 3488 cm^{-1} for the strong vibrations for the primary amines describes the complete mineralization of RBHEBL.

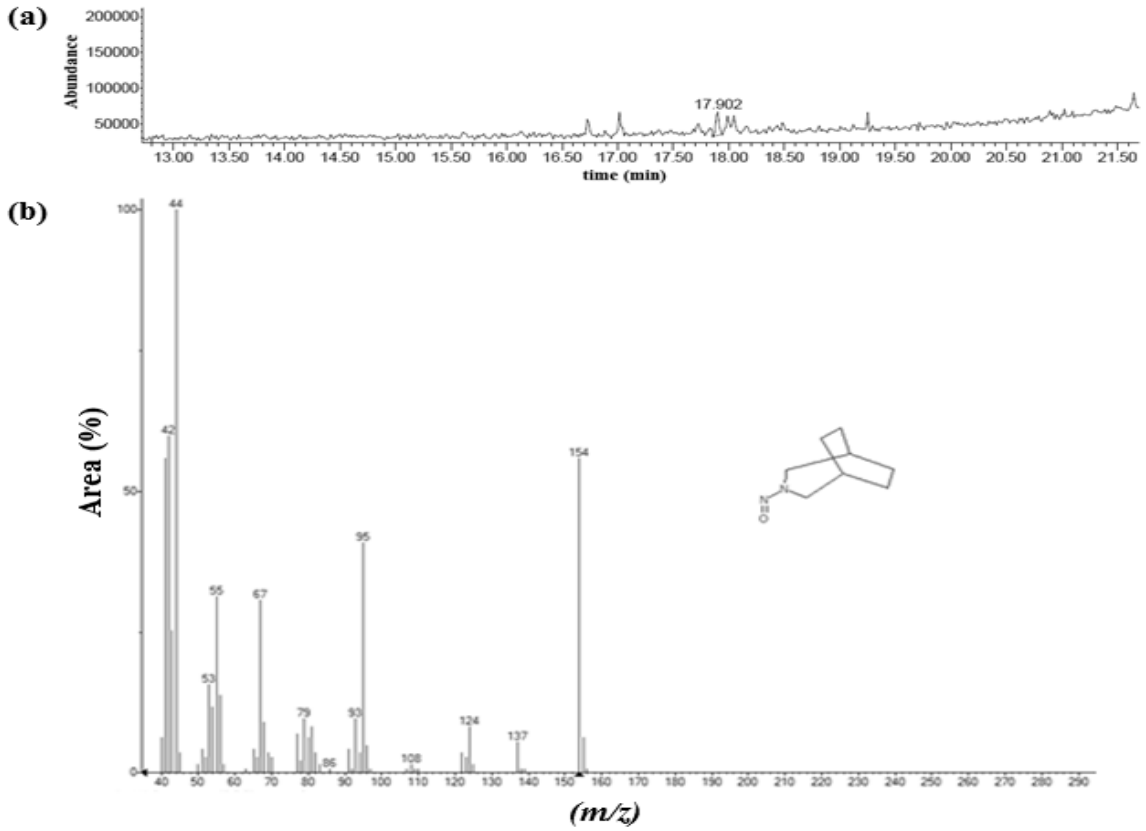
The HPLC elution profiles of RBHEBL before and after treatment with the mixed culture show that the dye peak (retention time 4.283 min) disappeared while three new peaks emerged with considerably shorter retention times (2.625, 3.525 and 4.292 min) as shown in the (Figure- 3) indicating the formation of more polar oxidation products. HPLC was used as a tool to monitor formation of degradation products obtained after treatment with the mixed culture. The HPLC analysis and the irreversibility of the dye removal indicate that further degradation contributes to dye removal in the case of the dyes tested.

Figure 3: HPLC chromatogram of (i) control RBHEBL and (ii) extracted metabolites



The mass spectra of the extracted metabolites eluted out of the column at 17.9 min with a peak area of 100%. It is evident that the parent RBHEBL compound was degraded into 3-nitroso-3-azabicyclo [3.2.2] nonane a product with a molecular formula, $C_8H_{14}N_2O$ and the molecular weight is 154 by the formulated bacterial consortium as shown in the (Figure- 4).

Figure 4: Mass spectral profile for the degradation of RBHEBL



Phytotoxicity assessment

Despite the fact that, untreated dyeing effluents may cause serious environmental and health hazards, they are being disposed off in water bodies and this water can be used for an agricultural purpose in India. The use of untreated and treated dyeing effluents in agriculture has a direct impact on the fertility of the soil. Thus, it is of concern to assess the phytotoxicity of the dye before and after degradation. The relative sensitivity of

Triticum aestivum and *Phaseoulus mungo* towards RBHEBL and their degradation product obtained were studied[22]. Germination inhibition increases linearly with the concentration of control and the extracted metabolites applied. The relative sensitivity of the plant seeds against control concentration and the extracted metabolites (500 mg/L) were evaluated. The seed germination percentage, length of plumule and radicle and the chlorophyll content of *Triticum aestivum* and *Phaseolus mungo* seedlings were significantly affected as compared to water (control set). There was an inhibition of germination and slower rate of plumule and radicle emergence and also reduced chlorophyll content of the seedlings at the control, whereas the extracted metabolite showed reverse phenomena as shown in (Table- 1).

Table 1: Phytotoxicity of RBHEBL (500 mg/L) and its extracted metabolites for the *Triticum aestivum* and *Phaseolus mungo*

Parameters	*Triticum aestivum*			*Phaseolus mungo*		
	Water	**RBHEBL**	**Extracted metabolites**	**Water**	**RBHEBL**	**Extracted metabolites**
Germination percentage (%)	100	60	85	100	70	90
Plumule length (cm)	18.12±0.72	8.5	8.75	18.1±0.45	5.9	9.7
Radicle length (cm)	16.96±0.33	3.75	3.75	7.8±0.39	1.0	3.8
Total chlorophyll (mg/g tissue)	0.027	0.010476	0.045289	0.015	0.00852	0.02396

The decrease in chlorophyll content of the plants in response to applied dye could be associated with increase in chlorophyllase, an enzyme responsible for chlorophyll degradation or decrease in the endogenous cytokinins involved stimulating chlorophyll synthesis. Increase in the abscissic acid (ABA) content in the leaves have to inhibit chlorophyll synthesis. As chlorophyll content is related to the growth of a plant, a decrease in their content indicates the toxic nature of the dyes. The decrease in the chlorophyll content also may be due to the destabilization of the chloroplast. The elongation of plumule and radicle and also the rise in the concentration of chlorophyll content in the case of extracted metabolites may be due to the increase in carbon and nitrogen sources. The outcomes of phytotoxicity studies indicate that the control dye compound was toxic and the metabolites were non toxic to crop plant seeds.

Conclusions

In this work, the biotransformation of RBHEBL was investigated using an isolated and acclimated mixed culture. The decolorization was confirmed by UV-vis spectrophotometric whereas FT-IR the spectroscopy along with chromatographic techniques like HPLC and GC-MS analyses revealed RBHEBL biotransformation into 3-nitroso-3-azabicyclo (3.2.2) nonane by the mixed culture. The induction of the LiP described the involvement of enzyme during RBHEBL biotransformation which drastically diminished COD. Finally, the extent of detoxification was evaluated using phytotoxicity assays, thereby confirming the reduced toxicity of RBHEBL.

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