Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2021.27246

226 (2021) 382–399
June

Synergistic effect of bacterial-fungal consortium for enhanced degradation of azo dyes – a novel approach

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Received 15 September 2020; Accepted 11 March 2021

ABSTRACT

In the present study, a defined bacterial-fungal consortiums PS-PAAT was developed to increase the production of the oxidoreductive enzymes for the effective and faster degradation of stable azo dyes. Aerobic batch degradation of azo dyes by both suspended growing cells and immobilized microbial consortiums were also investigated in this study. Interesting, the activity of the MnP and laccase were significantly higher for immobilized consortium than for free suspended cells, while azoreductase activity was found to be lower in immobilized consortium than the free cells. The reusability and stability of the immobilized consortium were successfully evaluated for the five repeated-batch decolorization experiments. Under the optimized conditions (pH-6, temperature 30°C, glucose 2 g L^{-1}, sodium nitrate 1.5 g L^{-1}, HBT 0.1 mM) the immobilized microbial consortium able to degrade more than 90% of selected azo dyes (150 mg L^{-1}) at 150 h of incubation in the cycle 1. After five repeated batch cycles, the decolorization rate of the free cells decreased by nearly 30%–50%, while immobilized cells still retained 80%–95% of their original activity. A developed immobilized consortium PS-PAAT completely decolorized Ponceau and Naphthol Blue Black under the static condition with an average decolorization rate of 7,500 and 9,375 μg h^{-1}, respectively; which is much faster than that of the free suspended cells. Further, the biodegradation was also monitored by Fourier transform infrared spectroscopy, high performance liquid chromatography, and gas chromatography–mass spectrometry analysis. Additionally, the study was also conducted to identify the intermediates formed during the degradation of azo dyes and their possible molecular metabolic pathways were proposed according to the results of metabolites identification and related literature. The results of toxicity studies suggest that this consortium may effectively be used for complete detoxification of dye and has potential environmental implications in cleaning up azo dyes containing effluents.

Keywords: Bacterial-fungal consortium; Biodegradation; Azo dyes; Oxidoreductive enzymes; Toxicity assay

1. Introduction

Over the past decades, due to the high demand for textile fabrics, there is a rapid increase in the production rate of dyestuff and applications of synthetic dyes. In worldwide, every year approximately 280,000 tons of dyestuffs are discharged into the aqueous environment as an effluent [1]. In most of the synthetics dye manufacturing industries, azo dyes are extensively used as dyestuff and also they are employed as substrates for making textile fibers, leathers, plastics, papers, foodstuff, and cosmetics [2]. Azo dyes are xenobiotic compounds and highly recalcitrant due to the presence of one or more azo groups (–N=N–) and sulfonic ($–SO_3^-$) electron-withdrawing group that can

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resist the breakdown and accumulates in the environment at high levels with a high degree of persistence [3]. The pollution caused by the mixture of dye-containing effluents is becoming increasingly alarming worldwide. Further, the release of dye-containing wastewater from the textile industries into the aqueous environment is undesirable and causes severe environmental damage [4,5]. Therefore, the treatment of azo dyes containing wastewater and their metabolites is an essential factor to eradicate the pollution before the discharge of wastewater into the environment.

Conventional physicochemical methods failed to achieve satisfactory results for dye removal because these methods are inefficient, expensive, have limited applications and produce a huge amount of wastes in the form of sludge, which again need to be disposed off [6]. Hence, there is an urgent need for all the researchers to develop an alternative technology for the complete mineralization of azo dyes. Recently, extensive research and development have focused on biological treatment methods due to their cost-effectiveness, easy availability, rapid degradation with less sludge formation and eco-friendly nature [7]. In order to achieve much effective degradation and decolorization in bioprocess technology for the treatment of azo dye-containing wastewater, it is the prime necessity to identify the capable microorganisms possessing high degradation efficacy of azo dyes. To make this concern, bacteria and fungi have been studied for the decolorization and degradation of various azo dyes. Selection of the indigenous adaptive microbial strain and strain development for the exploitation of their enzymes with different substrate specificity is also considered as one of the prime factors for complete mineralization of mixed dyes [8,9]. In the recent past, several microorganisms have been used as an effective tool for the decolorization process due to their strong adaptability nature and rapid decolorization efficiency. Unfortunately, so far there is no individual microbial strain has been reported for degradation of mixed azo dyes completely and further, the intermediate compounds are released during the degradation process of individual strains are often carcinogens, which need further decomposition by the other strains [10]. The mixed microbial consortia were suspected to have supremacy over pure isolates in treating azo dye-containing wastewater. It may be due to the co-metabolism activity of microbes or by the different oxidoreductive extracellular enzymes released by the microbes. The synergistic effect of bacterial-fungal consortium in bioremediation technology offers several advantages as some strains can utilize the toxic metabolites produced during the degradation process which leads to complete mineralization of azo dyes. Nowadays, in bioprocess technology, the immobilized microbial cell has been suggested widely for protecting the microbial cells from the toxic metabolites generated during the degradation process or variations in the environmental conditions [11]. Immobilization of microbial cells has been apparently used in a biodegradation process when compared to the free cells due to the high cell density, cost-effectiveness, operation stability, easy recovery, and reuse [12–14]. Among the entire immobilizing agent, natural gel such as alginate has been widely used in cell entrapment techniques due to its nontoxicity nature, high performance, and greater substrate diffusion ability [15]. In this study, a novel defined bacterial-fungal consortium was developed and immobilized by entrapment in the sodium-alginate-gel matrix. The nine model dyes were selected from the previous study used for investigating the effect of different parameters on degradation by suspended bacterial-fungal cells and the immobilized cells. Additionally, oxidoreductive enzyme activity was calculated from the developed microbial consortium and the fate of degradation pathways was also analyzed. To my adequate knowledge, this may be the first report for the over-production of oxidoreductive enzymes and their efficiency in rapid decolorization of model dyes by the immobilized bacterial-fungal consortium.

2. Materials and methods

2.1. Dyestuff and chemicals

The environmental stable (pH, temperature, and intensity of light) azo dyes with color index (CI) number such as Acid Blue 113(AB 113) (CI-26360), Naphthol Blue Black (NBB) (CI-20470), Congo Red (CR) (CI-22120), Ponceau (P) (CV-27195), Tartrazine (T) (CI-19140), Reactive Orange 16 (RO 16) (CI-17757), Reactive Black 5 (RB 5) (CI-20505), Trypan Blue (TB) (CV-23850), and Disperse Blue 106 (DB 106) (CV-111935) were selected from the previous study and they were used for these experiments. The azo dyes used for this study were of a high standard with analytical grade. The remaining chemicals used in these experiments were obtained from Sigma Aldrich, India.

2.2. Microbiological media for cultivation and preservation of microbes

Nutrient broth (NB) and potato dextrose broth (PDB) were used for the preservation of bacterial and fungal cultures respectively. Dye degradation studies were carried out using the modified Kirk's basal salt medium according to the composition stated earlier by Saroj et al. [16]. All the medium used for the experiments were adjusted to pH 6.8 and sterilized by using autoclave at 121°C for 15 min and used for further studies.

2.3. Isolation, identification, and selection of potent azo dye degrading microorganism

The mutated microbial strains such as *Pleurotus ostreatus* MTCC 142, *Trametes hirsuta* MTCC 1171, and *Pseudomonas* B1 strain obtained from the previous study were used for this experiment [17–19]. *Stenotrophomonas maltophilia* AK2, *Aspergillus terreus* AKF2, and *Aspergillus tubingenesis* F1 were isolated through enrichment techniques from the dye-polluted area (Tiruppur region, India) and selected based on their ability to degrade azo dyes. These bacterial and fungal isolates were identified using 16s rDNA and 18s rDNA sequence analysis, respectively, according to Amasha et al. [20] and Yang et al. [21]. The 16s rDNA of *Stenotrophomonas maltophilia* AK2 and 18s rDNA sequence of *Aspergillus terreus* AKF2 and *Aspergillus tubingenesis* F1 were submitted to the nucleotide bank database and obtained the accession number KJ685809, KJ685810, and KM275619, respectively. All the isolated and mutated strains were cultivated individually

in 500 mL conical flasks containing the culture broth at 37°C for 24–48 h of incubation in a rotary shaker (100 rpm) and used for the degradation studies.

2.4. Bacterial-fungal consortia

Bacterial consortia-PS was prepared by transferring 1 mL of each bacterial culture suspension (10^4–10^5 cells mL^{-1}) of *Pseudomonas* B1 and *S. maltophilia* AK2 into conical flasks containing 100 mL of nutrient broth, spiked with 50 μL of all selected azo dyes and then incubated at 37°C for 96 h under aerobic conditions (shaking at 120 rpm). The fungal consortia-PAAT was developed by transferring 1 mL (10^7–10^8 spores mL^{-1}) of each fungal strain *P. ostreatus* MTCC 142, *A. terreus* AKF2, *A. tubingenesis* F1, and *T. hirsuta* MTCC 1,171 into 250 mL conical flasks supplement with 100 mL of PDB, spiked with 50 μL all selected azo dyes. All the flasks were incubated in the rotary shaker (150 rpm) at 30°C for 96 h under aerobic conditions. Finally, the bacterial-fungal consortium PS-PAAT was prepared by adding equal concentration (w/v) of bacterial and fungal grown culture in a sterile 500 mL conical flask. The newly developed bacterial-fungal consortium was used as inoculums for the degradation studies.

2.5. In-vitro compatibility assay of microbial consortium

A dual culture assay was performed to determine the antagonist activity between the six selected microorganisms based on the growth rate of two strains in the nutrient agar medium. The bacterial colonies or fungal mycelia (3 mm in diameter) were placed on one side of the medium whereas the other strains (bacteria or fungi) were transferred to the same medium 5 cm away from each culture. The single strain was inoculated in the medium as a control. The control and dual cultures were kept in the incubator at 30°C for 3 d. The experiment was repeated twice with three replications of each treatment. The percent growth inhibition (PGI) was calculated using the formula:

$$\mathrm{PGI}(\%) = \frac{C - M_1}{C} \times 100 \tag{1}$$

where, C denotes that the distance (measured in mm) covered by the colony margin from the point of inoculation on the control dishes, and M_1 represents the distance covered by the microbial growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist [22]. After the end of the incubation period, the zone of inhibition was calculated by measuring the distance between the two microorganisms and categorized based on a growth inhibition category (GIC) scale [23].

2.6. Interaction study of microbial community

Microbial interaction study was carried out separately for each selected bacterial and fungal strains on composite broth medium (CBM) according to the procedure described by Mishra et al. [24]. The experiment was conducted in two steps. In the first step, the bacterial cell (10^6 cells mL^{-1}) or fungal spore (10^4 cells mL^{-1}) were aseptically transferred to the CBM and incubates at 35°C in a rotary shaker (120 rpm) for 72–120 h. After obtaining the sufficient growth pattern in the medium, the cell mass was separated through filtration using Whatman No. 1 filter paper. Then, the filtrate was sterilized using autoclave at 121°C for 20 min. In the second step, the equal amount of sterile filtrate was amended with CBM and inculcated with the other selected microorganisms. All the tubes were kept in an incubator at 35°C in a rotary shaker (120 rpm) for 72–120 h. The different combinations of microbial strains were developed to understand the positive and negative interactions among the selected microorganisms. Microbial interaction study was carried out separately for each selected bacterial and fungal strains and the relative growth conversion (%) was calculated using the equation:

$$\text{Relative growth conversion}(\%) = \frac{B_2 - B_1}{B_1} \times 100 \tag{2}$$

where B_1 is the biomass of individual bacterial or fungal strain in broth and B_2 is the biomass of the same bacteria or fungal strain in the spent filtrate of other strain amended with culture broth after 72 h for the bacterial community and 120 h for the fungal community.

2.7. Immobilization of microbial consortium

The exponentially growing culture of bacterial consortium-PS and biomass from 10 to 12 d old cultures of fungal consortium-PAAT were collected by the centrifugation process. The pellet was washed with sterile water and then suspended with the phosphate buffer solution to make an optical density (OD) to 1.8 at 560 nm, which was equivalent to a cell concentration of 0.162 g L^{-1} (dry weight). Sodium alginate was used as the immobilizing agent for bead preparation. The collected bacterial and fungal cultures were resuspended in 100 mL sterile millipore water and mixed thoroughly to get a final concentration of (4%, w/v) alginate solution. The thoroughly mixed bacterial-fungal cell-alginate solution was taken with the different types of the sterile syringe and allowed to fall in 250 mL calcium chloride solution (0.1 M) drop by drop for beads formation at 4°C, for 15 min to obtain a permanent shape. The different size of beads was collected and kept in the same solution for 30 min at 4°C for hardening. The resulting beads were washed twice with phosphate buffer (pH 7.0) and subsequently with distilled water to remove excess Ca^{2+} ions and unentrapped cells.

2.8. Oxidoreductive enzyme assay

All oxidoreductive enzyme assays were performed spectrophotometrically (LAMBDA 1050 UV-vis spectrophotometer, Perkin Elmer, India). MnP activity was calculated based on the oxidation of guaiacol (2-methoxyphenol) according to the procedure described by Mansur et al. [25], whereas the laccase activity was calculated based on the oxidation of ABTS [26] and also azoreductase activity was determined using the azo dyes as substrates [27]. One unit of (U) enzyme activity is defined as the amount

of enzyme required to reduce 1 μmoL of substrate min^{-1} under the assay conditions. All reactions were performed in triplicate.

2.9. Optimization parameters for accelerated degradation of azo dyes

In order to achieve maximum dye degradation rate by the developed microbial consortium, the experiments were performed in 250 mL sterilized conical flask containing 100 mL sterilized Kirk's medium under different culture conditions using traditional stepwise protocol, that is, changing a factor at a time and keeping others constant for the optimization of dyes concentration (50, 100, 150, and 200 mg L^{-1}), pH (4, 5, 6,7, and 8), temperature (10°C, 20°C, 30°C, and 40°C) and concentration of glucose (1, 2, 3, and 4 g L^{-1}), the concentration of sodium nitrate (0.5, 1, 1.5, and 2 g L^{-1}) and concentration of redox mediator such as 1-hydroxybenzotriazole (HBT) (0.05, 0.1, 0.15, and 0.2 mM) was added to check the efficacy of azo dye decolorization rate under previously optimized conditions. All the culture flasks were kept in a rotary shaker (150 rpm) until the complete decolorization occurs in the medium.

2.10. Decolorization assay

The batch decolorization experiment was conducted separately for the freely suspended bacterial-fungal cells and immobilized consortium PS-PAAT in a 250 mL sterile conical flask containing 100 mL sterilized Kirk's medium amended with azo dyes (150 mg L^{-1}) under optimized conditions. The decolorization reaction was initiated by adding 3 g of beads or equivalent biomass of freely suspended bacterial and fungal cells into the medium. In order to obtain the complete agitation, all the experimental flasks were placed in a rotary shaker for 150 rpm for 0–150 h. The supernatants were collected from the medium at regular intervals and then analyzed UV-spectrophotometrically for each model dyes at a specified wavelength. After the complete decolorization of dyes, the cell pellets were harvested from the culture medium containing freely suspended cells through centrifugation (12,000 rpm for 15 min); while in immobilized cells, the beads were removed by using Whatman filter paper, then washed with the buffer solution (pH-7) and transferred to the fresh medium for the second batch study. The batch experiment was carried out repeatedly for the five cycles. The percentage of decolorization was calculated according to Saratale et al. [28,29] were follows:

$$\text{Decolorization } \% = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100 \quad (3)$$

The average decolorization rate (μg h^{-1}) was calculated based on the method of Jadhav et al. [30] were follows:

$$\text{Average decolorization rate} = \frac{C \times \%D \times 1{,}000}{100 \times t} \quad (4)$$

where C is the initial concentration of dye (mg L^{-1}) and $\%D$ is the dye decolorization (%) after time t (h).

2.11. Characterization of degraded metabolites

The metabolites obtained after the complete degradation of model dyes were collected from the kirk's medium using a membrane filter (0.45 μm) for freely suspended cells and Whatman filter paper for immobilized cells. Then the extracted metabolites were mixed with an equal volume of ethyl acetate. The extracts were air-dried and evaporated to dryness in a rotary evaporator. The dried extract was then dissolved in a few volumes of high performance liquid chromatography-grade (HPLC) methanol and was used for further analysis. Fourier transform infrared (FT-IR) analysis was done on Shimadzu 8400S spectrophotometer (Shimadzu HPLC LC20AD, Shimadzu Pvt., Ltd., Japan) in the mid-IR region of 400–4,000 cm^{-1} with 16 scan speed. The samples were prepared using spectroscopically with pure KBr in the ratio 5:95; pellets were fixed in the sample holder and analyzed. The dye degradation was monitored by HPLC (Shimadzu HPLC LC20AD, Shimadzu Pvt., Ltd., Japan) as the decolorization continued. HPLC analysis was performed in an isocratic Waters 2690 system equipped with a dual absorbance detector. Column specifications were a C18 column (symmetry, 2.1 mm × 150 mm) with particle size 1.5 μm. HPLC grade methanol: water (80:20) was used as the mobile phase with a flow rate of 1 mL min^{-1} and HPLC run was carried out for 10 min. The GC-MS analysis of metabolites was carried out using Agilent GC system 7890 A, MS 5975 C (Triple Axis Detector) with integrated gas chromatography with DB 35MS Column J&W 122-3832:340°C: 30 m × 250 μm × 0.25 μm. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The injector was maintained at 220°C with oven condition as 60°C for 1 min and increased up to 200°C with 15°C min^{-1} to 310°C for 5 min.

2.12. Toxicity assay

2.12.1. Phytotoxicity test

Phytotoxicity test was carried out using two different seeds of commercially available plants such as *Vigna mungo* and *Sesamum indicum*. The phytotoxicity of the untreated dye and treated dye solution was assessed by the seed germination of *V. mungo* and *S. indicum*. The seeds were selected as it is one of the major and widely consumed perennial plants of India. The model dyes (500 mg L^{-1}) and the obtained metabolites were separately dissolved in a 500 mL conical flask containing the sterilized distilled water. The two selected seeds were taken and sterilized with $HgCl_2$ (1%) and were rinsed thoroughly with distilled water. The seeds were sowed into a plastic sand pot with daily watering of 10 mL of dye solutions or its degradation metabolites obtained after degradation by immobilized cells. The control set was carried out using distilled water at the same time. Germination index was calculated according to the root was excised from the cotyledons and the length of the root was calculated after 20 d.

2.12.2. Microbial toxicity test

The bacterial toxicity assay was conducted using two bacterial strains which are responsible for improving soil fertility [31]. The selected bacterial cultures were made to

grow on Mueller and Hinton agar plates. Four wells of 6 mm in diameter were made in each plate with a sterile borer and seeded with the products obtained after dye degradation. The plates were incubated inappropriate conditions and the zone of inhibition was calculated to measure the effect of toxicity. The data represent the mean and standard deviation of the three experiments.

2.13. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Turkey–Kramer multiple comparison tests.

3. Results and discussion

The bacterial-fungal consortium used for dye degradation studies was designated as PS-PAAT and contained two bacterial (*Pseudomonas* species B1 and *S. maltophilia* AK2) and four fungal strain (*P. ostreatus* MTCC 142, *A. terreus* AKF2, *A. tubingenesis* F1, and *T. hirsuta* MTCC 1171) which have been selected from the previous study based on the enzyme production and their ability to degrade the selected azo dyes.

3.1. In vitro antagonist activity of selected bacterial and fungal strain

To develop an efficient bacterial-fungal consortium for potential degradation of azo dyes, it is essential to check the antagonist activity of each microorganism because every microorganism is capable of producing different diffusible metabolites, volatile organic compounds (VOCs) or toxins, which directly or indirectly inhibit the growth of microorganisms and rate of degradation. Keeping this in view, the dual culture method was performed to analyze the compatibility of selected microbial strains. All the selected bacterial and fungal strains displayed a less antagonistic activity (>25%) among themselves. Further, among the six microbes selected, *T. hirsuta* showed a low level of antagonistic activity toward the strain *Pseudomonas* B1 with a low PGI value (21%), followed by *S. maltophilia* (19%), whereas the remaining strains exhibited very less (>20%) antagonist activity against the selected strains (Fig. 1). A previous study reported that the antagonistic activity of microbes was depending upon the factors such as type

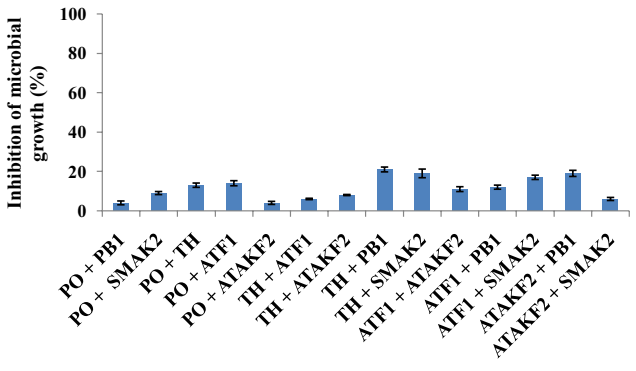


Fig. 1. Determination of *in-vitro* antagonist activity of bacterial and fungal strain by dual culture assay.

of the strain, host-specificity, and the culture media used in the experiment. The isolated strain *G. roseum* showed a low PGI value (21%) against the pathogens *C. gloeosporioides*, while *S. noursei* have 82% [22]. The results of the dual culture assay revealed that all the potent azo degrading strains belong to the GIC 1. Hence, the developed consortium may able to grow efficiently in the culture media and capable of degrading the model dyes very effectively.

3.2. Microbial interaction study

A microbial interaction study was also conducted to examine the positive or negative interaction of the developed microbial community in terms of relative growth conversion based on metabolite released during the reaction. The growth of *P. ostreatus* was decreased by 3.4% and 8% in presence of *T. hirsuta* MTCC 1171 and *A. terreus* AKF2 spent filtrates, respectively. On the other hand, *P. ostreatus* growth was enhanced more than 5% in all the remaining spent filtrate. Similarly, the spent filtrate of other selected microorganisms greatly enhance the growth of most of the microorganism and has a less negative impact (>20%) on the growth of remaining microbes (Fig. 2). In view of above observations, it was elucidated that the developed bacterial-fungal community does not have much effect on their metabolite produced by themselves. The suppression of the microbial community occurs due to the change in the medium, pH, temperature, and also depends on the relative growth rates of the individual species [32].

3.3. Effect of calcium-alginate beads size on decolorization of model dyes

In order to improve the dye degradation efficiency in wastewater using microbial consortium, a suitable bioreactor should have to be developed in which microbial flora can grow and survive for a long period with high degradation efficiency. A promising advanced technology to acquire this goal is the immobilization of bacterial-fungal consortium cells in/on an appropriate biopolymer matrix. Further, the choice of entrapment matrix and the size of the beads play a vital role in the efficient decolorization of azo dyes by the immobilized cells. In most of the research, alginate has been extensively used as an entrapping agent for the microbial cells due to their higher operational stability, active diffusion ability of enzyme or substrate and also able to preserve the microbial cells in a viable condition for the long period [33]. In the present study, the different size of alginate beads ranging from 0.6 to 5.4 mm was prepared by using bacterial-fungal consortium to obtain the maximum degradation of selected model dyes. Upon 72 h of incubation at room temperature, the maximum decolorization was achieved in all selected model dyes (100 mg L^{-1}) when the bead size was kept 4.2 mm in diameter (Fig. 3). The rate of degradation was decreased when the bead size increases or decreases from the 4.2 mm diameter and this is due to the internal diffusion factors [34]. Furthermore, the maximum degradation efficiency was observed in methyl orange when it was treated with alginate-immobilized *Aeromonas* species at 2 mm bead size [35].

3.4. Optimization parameters for the accelerated degradation of azo dyes

In order to achieve the maximum decolorization of selected model dyes, the effect of different optimization parameters (pH, temperature, glucose concentration, sodium nitrate concentration, HBT concentration, and dye concentration) were examined for the immobilized bacterial-fungal consortium. The effect of pH on the decolorization of model dyes was carried out by varying the pH concentration in the range of 4–8. The consortium PS-PAAT showed the maximum degradation at pH-6 for most of the model dyes such as AB 113 (81%), NBB (89%), CR (88%), P (90%), T (79%), and TB (88%); while for the model dyes such as DB 106 (81%), RB 5 (83%), and RO 16 (53%) maximum degradation was achieved at pH-7 (Fig. 4a). In most of the literature survey, it was reported that the maximum color removal of azo dyes was observed in the range of pH 5–8 for the microorganisms *Trametes versicolor* [36], *Cunninghamella elegans* [37], *Rhizopus nigricans* [38]. However, in the extreme pH, the rate of degradation was decreased due to the reduction in microbial growth. Fig. 4b showed the effect of temperature on the decolorization of model dyes by the immobilized microbial consortium. It was noted that maximum degradation (<90%) was achieved for the model dyes selected was in the ranges of 30°C–40°C. From the results obtained, it was evident that the decolorization efficiency decreased when the temperature is increased above 40°C or decreased below 30°C. Figs. 4c and d showed the effect of glucose and sodium nitrate concentration, respectively, on decolorization of model dyes by developed bacterial-fungal consortium PS-PAAT. The results suggest that the selected model dyes exhibited maximum decolorization when the glucose concentration is 2% and sodium nitrate concentration is 1.5%. Further, it was obvious that when the concentration of glucose and sodium nitrate level increase or decrease

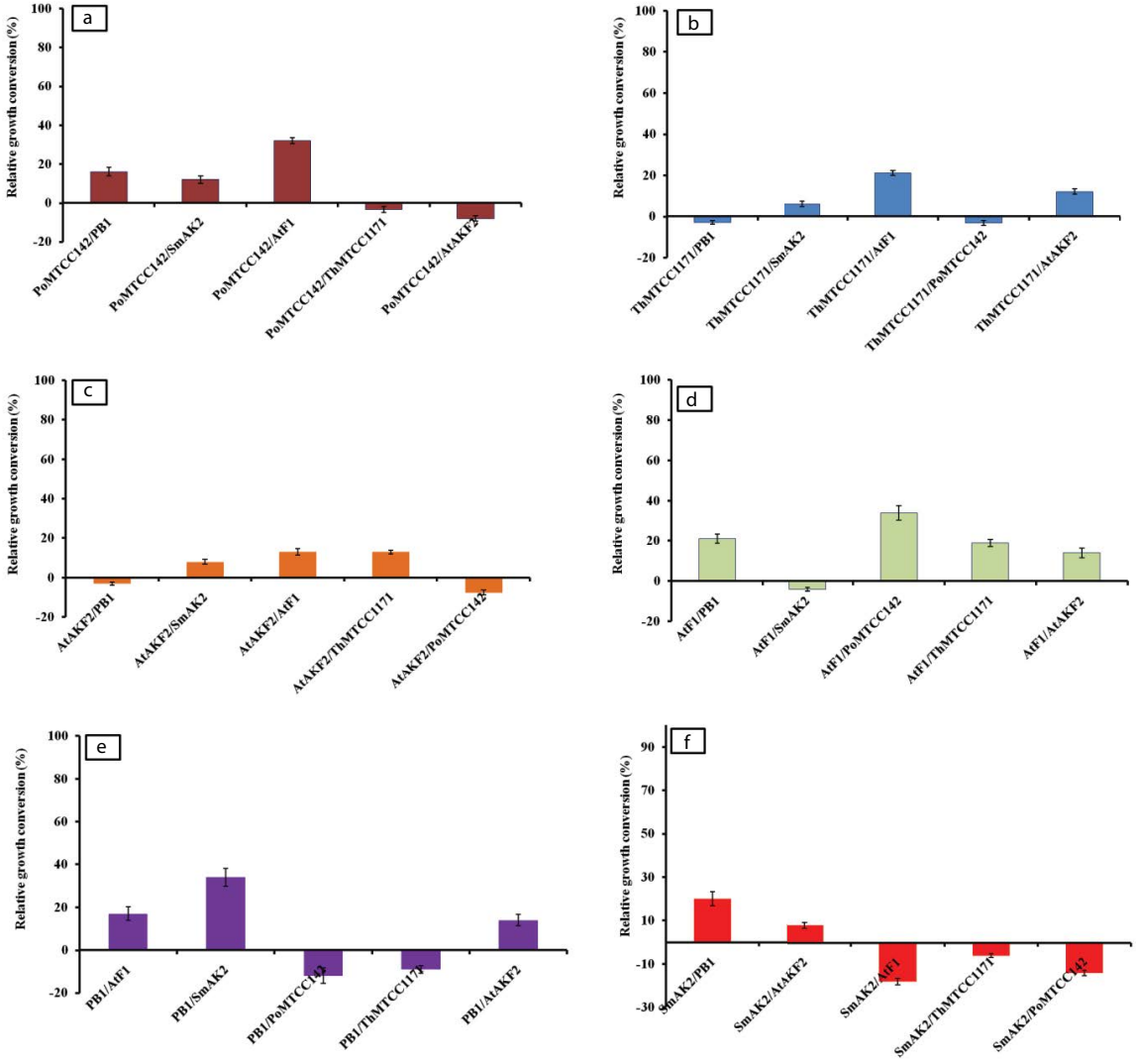


Fig. 2. Microbial interaction studies among the selected bacterial and fungal strains in terms of relative growth conversion in %. (a) *Pleurotus ostreatus* MTCC 142, (b) *Trametes hirsuta* MTCC, (c) *Aspergillus terreus* AKF2, (d) *Aspergillus tubingenesis* F1, (e) *Pseudomonas* B1 strain, and (f) *Stenotrophomonas maltophilia* AK2 growth in the presence of other microbial metabolites obtained after 120 h.

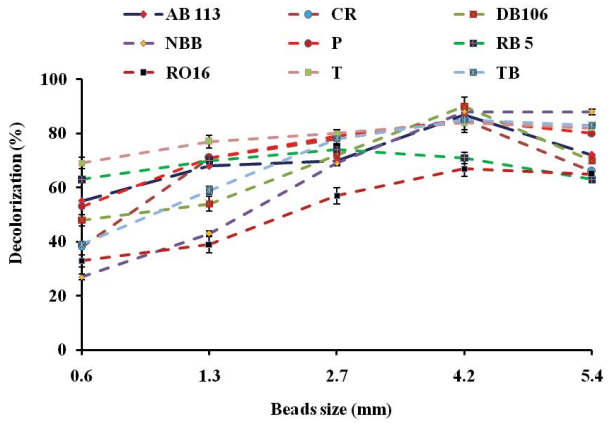


Fig. 3. Effect of sodium-alginate bead size on decolorization of model dyes (100 mg L^{-1}) by immobilized bacterial-fungal cells after 72 h of incubation at 37°C.

from the above corresponding ranges, the decolorization rate of dyes significantly decreases. In the present study, glucose, and sodium nitrate was used as carbon and nitrogen sources for the microorganisms to enhance the decolorization process by the way of co-metabolism. However, if the external source of energy molecules is given to the microorganism, the microbes preferentially utilize the substrates first to reach the saturated level and thereby increase the rate of degradation of dyes mainly due to the hyperproduction of oxidoreductive enzymes [39,40]. Generally, the redox mediator HBT enhances the degradation of dyes by increasing the catalytic activity of enzymes present in the microbes. The effect of different concentrations of HBT and dyes (50, 100, 150, and 200 mg L^{-1}) on decolorization of model dyes using immobilized bacterial-fungal consortium PS-PAAT were tested until the complete decolorization occurs in the medium and the results were depicted in Figs. 4e and f, respectively. In the presence of 0.1 mM HBT,

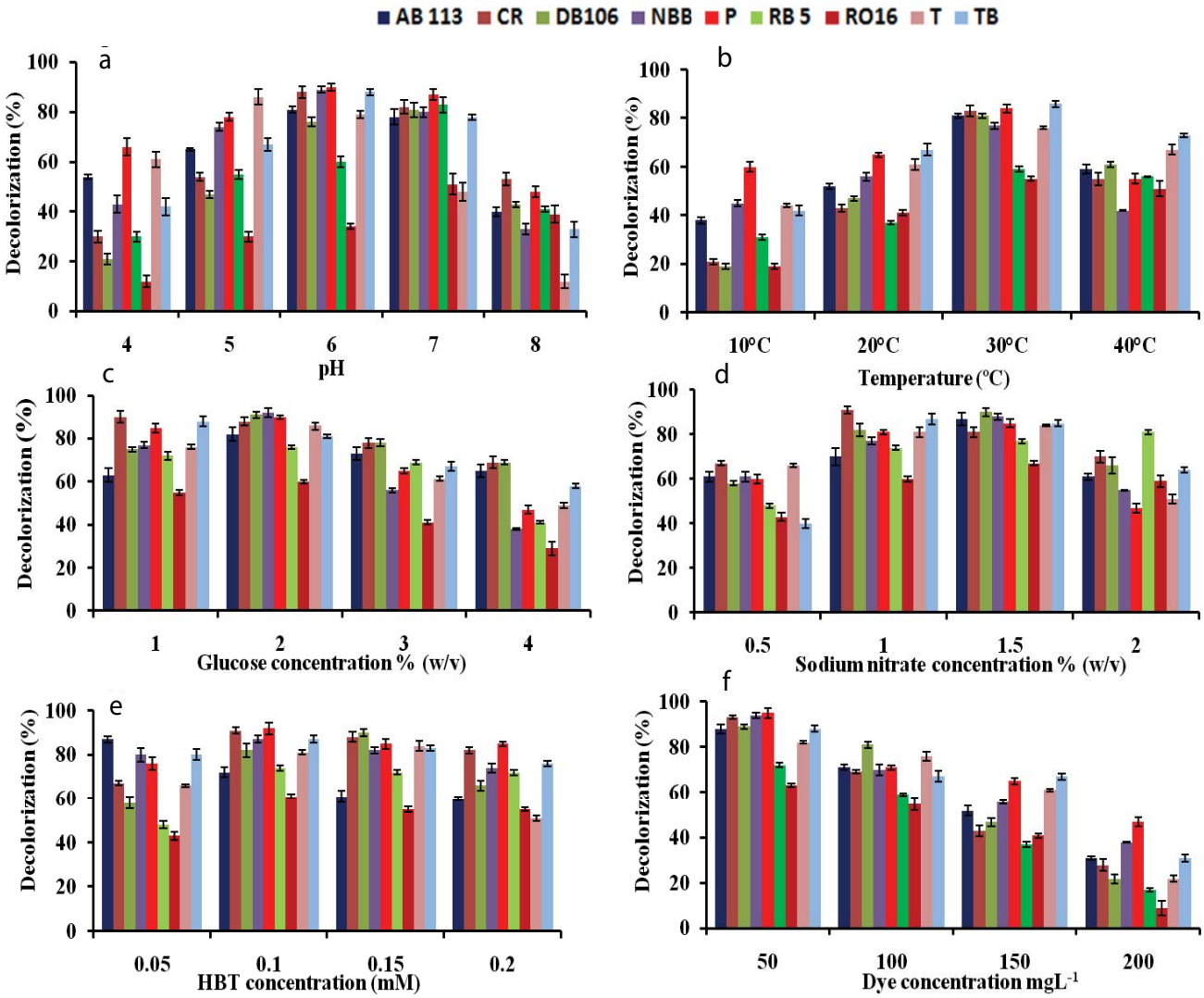


Fig. 4. Effect of different parameters including (a) pH, (b) temperature, (c) glucose concentration, (d) sodium nitrate concentration, (e) 1-Hydroxybenzotriazole (HBT) concentration, and (f) dye concentration on selected model dyes under aerobic conditions by the bacterial-fungal consortium after 72 h of incubation.

the immobilized bacterial-fungal consortium could effectively remove the color of most of the model dye. The present finding is found to be similar to the results of Sayahia et al. [41]. In many literature surveys, it was observed that the addition of redox mediator especially HBT enhanced the decolorization rate of recalcitrant synthetic dyes by fungal or by the enzyme treatment [42–44]. From the result obtained it was evident that the minimum concentration of HBT (0.1 mM) is enough for enhancing the decolorization activity of all model dyes. The rate of decolorization was increased with the initial dye concentration 50 mg L^{-1} and then gradually decreases when the concentration of dye increases. This effect may due to the structural complexity of the dyes, toxicity nature of the dye, absorption ability of the matrix used in the experiments, inhibition of microbial growth created by the adequate amount of dyes, and the reduction in the number of enzymes per unit volume of dye [45–47]. Further, our immobilized microbial consortium shows greater decolorization efficiency toward all the model dyes selected even in a high concentration of dyes and hence, it may be applicable to treat the mixed dyes present in the waste-water treatment of the textile industries.

3.5. Comparative analysis of decolorization of model dyes using free cell suspension and the immobilized consortium PS-PAAT

For the past two decades, it was noted that the microorganism and their enzymes were used as an effective tool in the field of bioremediation technology to eradicate the various dye pollution present in the textile effluents [48]. The ability of free cell suspension and immobilized cells to decolorize the various model dyes selected was evaluated in Kirk's medium under optimized conditions. Further, aerobic batch degradation of azo dyes by both suspended growing cells and the immobilized microbial consortium was also investigated in this study. Under the optimized conditions (pH-6, temperature 30°C, glucose 2 g L^{-1}, sodium nitrate 1.5 g L^{-1}, and HBT 0.1 mM) the immobilized microbial consortium showed a higher rate of decolorization (<90%) towards the selected model azo dyes (150 mg L^{-1}), except reactive orange 16 (78%) and disperse blue (86%) at 150 h of incubation, when to compare to non-immobilized cell (Fig. 5). Further, the immobilized consortium showed the maximum decolorization of about 98% and 99% toward the Naphthol Blue Black and Ponceau dyes, respectively. The higher decolorization efficiency of all model dyes by immobilized cells is due to the synergetic effect of bacterial and fungal consortium and the effect of their enzymes produced by microbes. However, microbial cell density in the gel matrix increases the quorum-sensing molecules that make the microbes to retain the stationary phase in the medium [49], thereby increases the enzyme production in it and thus leads to the complete mineralization of various dyes.

3.6. Repeated batch decolorization assay

The reusability and stability of the immobilized and non-immobilized consortium were successfully evaluated for the five repeated-batch decolorization experiments. It was obvious that after the end of five batch cyclic experiments, the degradation rate of immobilized cells increased by nearly 80%–95%, while free cells retained only 30%–50% of their original activity for the selected azo dyes (Table 1). The eventual reduction in decolorization rate was observed in the non-immobilized cells is due to the nutrient depletion or the direct toxicity effect of dyes on a cell [28]. In contrast, the immobilized consortium PS-PAAT was quite stable and able to decolorize (<80%) of the model dyes selected, even up to the fifth cycles under optimized conditions. Thus, the result indicates that the developed consortium may be applicable for the bioremediation technology to eradicate the mixed dye-containing wastewater, discharged from the textile industries.

3.7. Average decolorization assay

In order to improve the practical bioprocess technology and to know the decolorization efficiency of the consortium, the average decolorization rate was calculated for each selected model dyes by using a non-immobilized and immobilized consortium PS-PAAT. The average rate of decolorization of model dyes significantly higher in the immobilized consortium PS-PAAT than in the non-immobilized cells of bacteria and fungi (Table 2). The maximum average decolorization rate was observed in cycle 1 for all the selected model dyes (150 mg L^{-1}) by using an immobilized consortium. In the present decolorization study, the Ponceau and Naphthol Blue Black were efficiently decolorized by immobilized cells within 20 and 18 h, with a maximum average decolorization rate of 7,500 and 9,375 μg h^{-1}, respectively. Interestingly, 0.45–0.7 fold increased average decolorization rate was observed in all model dyes by the immobilized cells when compared with the non-immobilized cells. Further, the time required for the degradation process is found to be very less for immobilized cells rather than the freely suspended cells, it is purely due to the combined effect of microorganisms [50] and their quorum-sensing system produced by the densely populated microbes in the matrix, which increases the oxidoreductive enzyme production in medium. The present investigation clearly demonstrates that the complete mineralization of two dyes was achieved mainly due to the

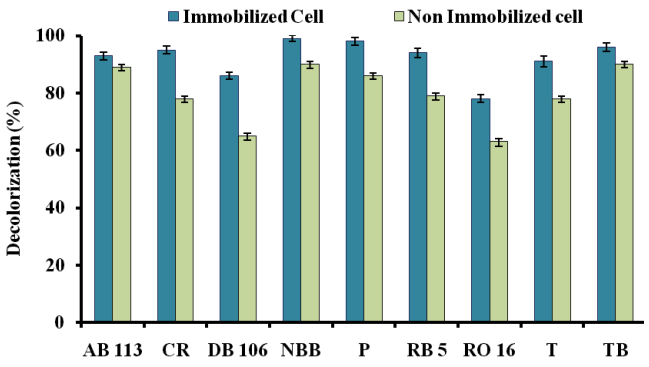


Fig. 5. Decolorization performances of immobilized and non-immobilized cells under optimized conditions after 150 h of incubation.

Table 1
Repeated batch decolorization assay of selected model dyes (150 mg L^{-1}) using non-immobilized and immobilized PS-PAAT consortium

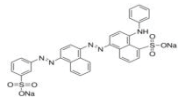
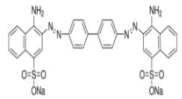
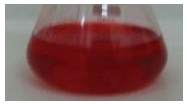
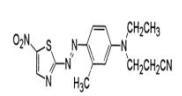
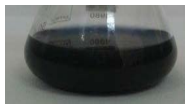
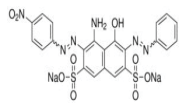
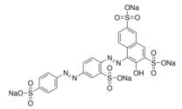
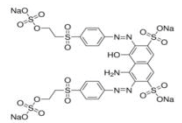
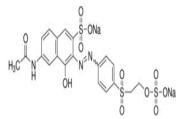
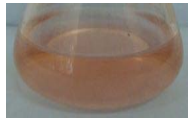
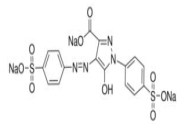
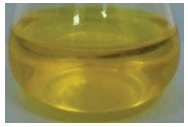
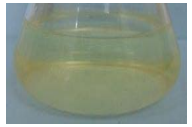
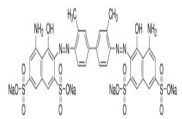
Azo dyes (λ_{max})	Structure of azo dyes	Before decolorization	After decolorization	Maximum decolorization in (%)					
				Non-immobilized cells	Immobilized cells				
					Cycle I	Cycle II	Cycle III	Cycle IV	Cycle V
AB 113 (566 nm)				89	93	90	87	80	73
CR (497 nm)				78	95	90	81	78	70
DB 106 (497 nm)				65	93	89	82	81	70
NBB (618 nm)				90	98	95	91	88	87
P (325 nm)				86	99	94	90	89	88
RB 5 (597 nm)				79	94	93	90	87	81
RO 16 (388 nm)				63	78	70	67	64	60
T (425 nm)				78	92	91	86	83	80
TB (607 nm)				90	96	93	90	86	86

Table 2
Average decolorization rate of immobilized and non-immobilized bacterial-fungal consortium in terms of (μg h^{-1}) on selected model dyes

Name of azo dyes	λ_{max}	Dye concentration	Non-immobilized cell	Average decolourization rate [ADR] (μg h^{-1})									
				Immobilized cell									
				Cycle 1		Cycle 2		Cycle 3		Cycle 4		Cycle 5	
		mg L^{-1}	Time (h)	[ADR]	Time (h)	[ADR]	Time (h)	[ADR]	Time (h)	[ADR]	Time (h)	[ADR]	Time (h)	[ADR]
AB 113	560	150	20	6,675	14	9,964	16	8,625	18	7,250	16	7,500	16	6,843
CR	497	150	19	6,157	17	8,382	14	9,642	17	7,147	16	7,312	17	6,176
DB 106	497	150	28	3,482	16	8,718	16	8,343	15	8,200	20	6,075	22	4,772
NBB	618	150	24	5,625	16	9,375	17	8,647	16	8,625	18	7,666	20	6,750
P	325	150	24	5,375	20	7,500	23	6,391	24	5,937	22	6,340	22	6,136
RB 5	597	150	29	4,086	18	7,833	18	7,750	20	6,750	21	6,214	24	5,062
RO 16	388	150	28	3,375	20	5,850	20	5,250	19	5,289	22	4,363	22	4,090
T	425	150	24	4,875	18	7,666	19	7,184	22	5,863	21	5,928	24	5,000
TB	607	150	25	5,400	14	10,285	16	8,718	16	8,437	17	7,588	18	7,166

two reasons such as the developed consortium used dyes directly as substrates (sole carbon source) or it needs suitable co-substrates (glucose, sodium nitrate, and HBT) for improving the rate of decolorization [51].

3.8. Enzyme assay

Currently, the oxidoreductive enzymes such as manganese peroxidase, laccase, and azoreductase produced by the various microorganisms pay much attention in a modern biotechnological process due to their wide substrate-specific activities. Thus, the analysis of extracellular enzyme activities released from the immobilized and non-immobilized cells is highly important for the complete mineralization of the azo dyes. For this reason, the activity of oxidoreductive enzymes was measured using the collected supernatants from the freely suspended and immobilized bacterial-fungal consortium in the presence of model dyes. It was observed that MnP activity was higher in the presence of Ponceau; the laccase activity higher in Naphthol Blue Black and azoreductase activity higher in the presence of Congo red. Further, the activity of the MnP and laccase were significantly higher for immobilized consortium PS-PAAT than from the freely suspended cells, while azoreductase activity was found to be lower in immobilized consortium than the freely suspended cells. Similarly, the protein content was also significantly higher in immobilized consortium PS-PAAT than from the freely suspended cells (Table 3). This result confirmed the presence of extracellular protein in the culture medium, especially the oxidoreductive enzymes used in the degradation process. Immobilization of microbial cells on several supporting agents increased the production of the oxidoreductive enzyme as compared to the freely suspended cells cultivated in the culture medium with the presence of dyes [52,53].

3.9. Microbial consortium mediated degradation analysis

The effectively degraded dye metabolites of Ponceau (99%) and Naphthol Blue Black (98%) by the immobilized consortium PS-PAAT were taken for the dye degradation analysis. The FT-IR spectrum of both control dye (Ponceau and Naphthol Blue Black) and the extracted metabolites of Ponceau and Naphthol Blue Black obtained after the degradation process by the immobilized consortium PS-PAAT showed a significant change in the positions of the peaks when compared to the control dye spectrum. The FT-IR spectrum of control dye Ponceau showed different peaks at 3,404; 2,890; 2,804; 2,522; 2,342; 2,095; 1,603; 1,453; 1,409; 1,088; 1,043; and 688 cm^{-1}. Similarly, the control spectrum of dye Naphthol Blue Black showed different peaks at 3,779.21; 3,471.08; 2,374; 1,614; 1,409; 1,164; and 684 cm^{-1} indicates the aromatic nature of the parental dye. The disappearance of the major peaks and the appearance of new peaks in the FT-IR spectrum of both the dye degraded metabolites suggest the biotransformation of dye into distinct metabolites. Specifically, the appearance of a new peak at 1,387 and 1,384 cm^{-1} represented C–H deformation of alicyclic CH_2, and the disappearance of the peak at 1,603 and 1,614 cm^{-1} on both the dyes metabolites gives clear evidence of azo bond breakage (Figs. 6 and 7). HPLC

Table 3
Estimation of total protein and oxidoreductive enzymes in the supernatants

Protein/oxidoreductive enzymes	Immobilized microbial consortium	Non-immobilized microbial consortium
Total protein (mg mL^{-1})	369 ± 3.3	226 ± 1.3
MnP activity (UL^{-1})	697 ± 4.9	578 ± 3.4
Laccase activity (UL^{-1})	51,201 ± 7.5	38,000 ± 5.2
Azoreductase activity (UL^{-1})	456 ± 5.7	555 ± 7.1

Values represent the means ± SD (n = 3), protein and oxidoreductive enzymes produced from the immobilized microbial consortium are significantly different from the non-immobilized microbial consortium at ($p < 0.05$).

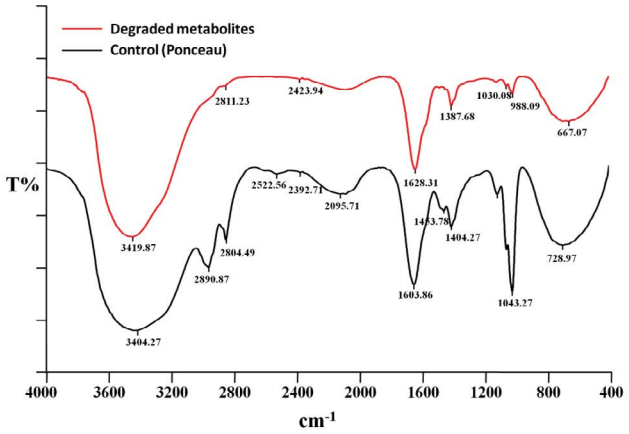


Fig. 6. FT-IR spectrums of control dye Ponceau and its metabolites obtained after the degradation by using immobilized bacterial-fungal consortium PS-PAAT.

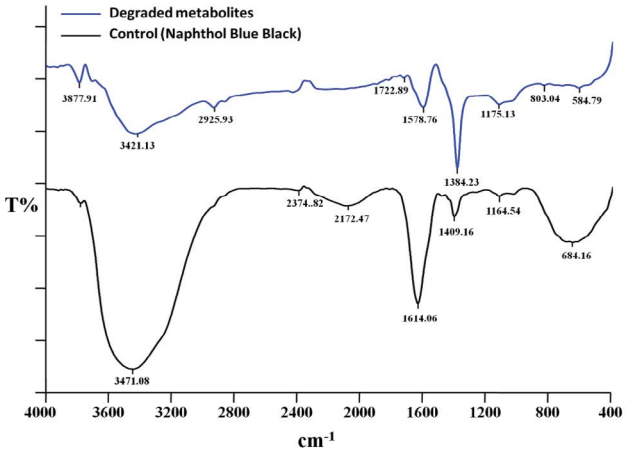


Fig. 7. FT-IR spectrums of control dye Naphthol Blue Black and its metabolites obtained after the degradation by using immobilized bacterial-fungal consortium PS-PAAT.

analysis confirms the biotransformation of Ponceau and Naphthol Blue Black mediated by immobilized bacterial-fungal consortium PS-PAAT. The control dye Ponceau showed a single major peak at a retention time of 2.048 min and two minor peaks at 2.946, 4.176 min (Fig. 8a), while the product obtained after the dye decolorization showed

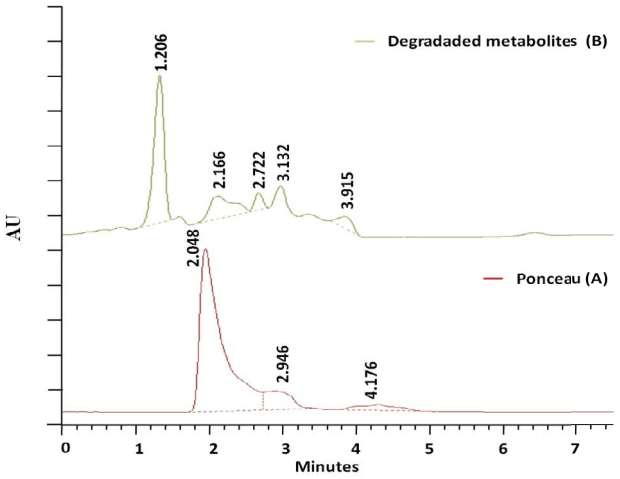


Fig. 8. HPLC chromatogram of control dye Ponceau (a) and its decolorized product obtained by using immobilized consortium PS-PAAT (b).

peaks at 1.206, 2.166, 2.722, 3.132, and 3.915 min (Fig. 8b). Moreover, the control dye Naphthol Blue Black showed a single major peak at a retention time of 1.602 min (Fig. 9a), while the decolorized product showed peaks at 1.371, 1.542, 1.781, 2.982, and 3.889 min (Fig. 9b), which was not present in the control dye. The formation of new peaks in the product of decolorized metabolites of both dyes and disappearance of the peaks seen in the control dyes indicates the degradation ability of the developed microbial consortium.

3.10. GC-MS analysis and proposed pathway of dye Ponceau and Naphthol Blue Black degradation by the immobilized consortium PS-PAAT

GC-MS analysis was performed to identify the degradation product formed after the end of decolorization assay of Ponceau and Naphthol Blue Black by the immobilized consortium PS-PAAT. The GC-MS chromatogram of degraded Ponceau metabolites showed an asymmetric cleavage of dye molecules with the primary reduction of an azo bond by azoreductase produced from the bacterial-fungal consortium yields four important metabolites such as benzene {78.11 m/z}, aniline {93.13 m/z}, naphthalene {128.17 m/z}, and naphthalene-1,3,6,8-tetrol {192.17 m/z} (Fig. 10). Similarly, the GC-MS analysis of degraded metabolites of Naphthol Blue Black showed various degraded

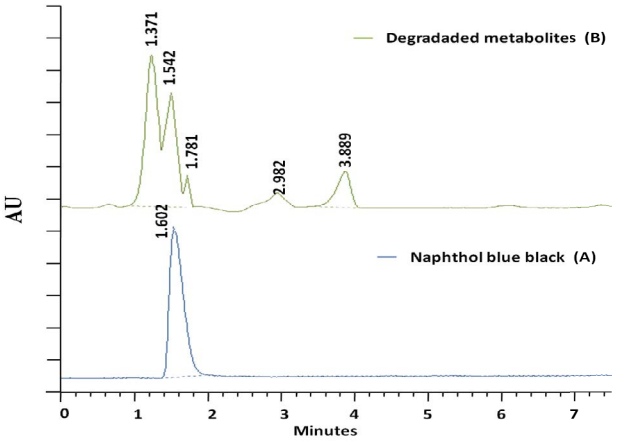


Fig. 9. HPLC chromatogram of control dye Naphthol Blue Black (a) and its decolorized product obtained by using immobilized consortium PS-PAAT (b).

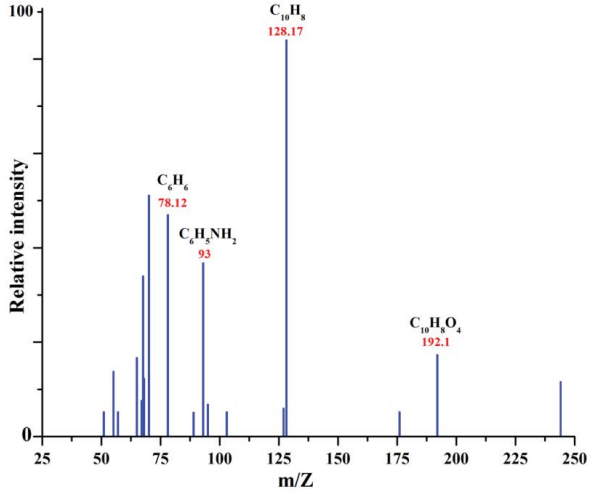


Fig. 10. GC-MS analysis of metabolites obtained after decolorization of Ponceau by immobilized consortium PS-PAAT.

products, which were confirmed by the presence of several peaks in the mass spectra (m/z) values. However, at the end of dye decolorization, five distinct metabolites such as benzene {78.11 m/z}, aniline {93.13 m/z}, naphthalene {128.17 m/z}, 4-nitroaniline {138.12 m/z}, and naphthalene-1,3,6,8-tetrol {192.1 m/z} were detected in GC-MS analysis along with several other metabolites due to the asymmetric cleavage of azo bond (Fig. 11). The proposed mechanism of Ponceau and Naphthol Blue Black degradation is depicted in Figs. 12 and 13, respectively. Rapid decolorization of dyes was observed mainly due to the cleavage of the chromophore group by the microbial enzymes produced from the consortium. Besides, complete mineralization and systematic degradation of dye molecules were achieved in both the dyes due to the hyperproduction of the oxidoreductive enzymes produced by the consortium with the initiation of azo reduction, deamination, desulfonation, and dehydroxylation. The laccase

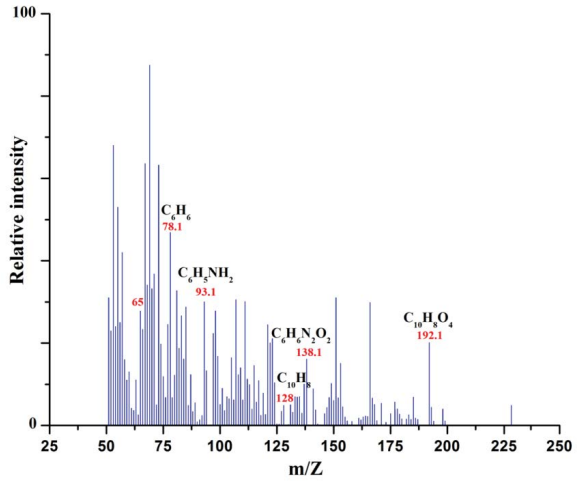


Fig. 11. GC-MS analysis of metabolites obtained after decolorization of Naphthol Blue Black by immobilized consortium PS-PAAT.

produced from *Pseudomonas* sp. SUK1 was responsible for oxidative asymmetric cleavage of azo dyes, whereas reductase was responsible for reductive cleavage of azo dyes [54,55]. As part of azo dye degradation, the formation of benzene and naphthalene has been previously reported by Lade et al. [56] and Phugare et al. [57].

3.11. Toxicity assay

The untreated dye-containing wastewater may cause severe environmental problems and also reduces the soil fertility if they are disposed of into stream water. To make this concern, the present study focused on assessing the toxicity effect of treated and untreated dye metabolites in plants and microorganisms. Results of the phytotoxicity assay revealed that the germination percentage of two selected plant seeds (*V. mungo* and *S. indicum)* showed 100% when irrigated with dye degraded products of Ponceau and Naphthol Blue Black, but the remaining selected dyes exhibited only 30%–90% of germination. Additionally, there is a significant level of changes was observed in the shoot and root length of both seeds treated with degradation metabolites or with water treatment when compared to untreated model dyes (Table 4). Similarly, in microbial toxicity assay, there was a no-zone formation occurs around the well against the Azotobacter *vinelandii* MTCC 2460 and *Pseudomonas putida* MTCC 2476 loaded by degraded metabolites of model dyes when compared with the untreated dye samples (Table 5). The toxicity assay suggested that the immobilized consortium completely detoxifies Ponceau and Naphthol Blue Black, while it also converts the remaining azo dyes to less toxic than untreated dyes.

4. Conclusion

A novel biodegradation approach with bacterial-fungal synergism was first applied for the degradation of stable azo dyes under static conditions. The immobilized microbial consortium PBS-PAAT exhibited broad

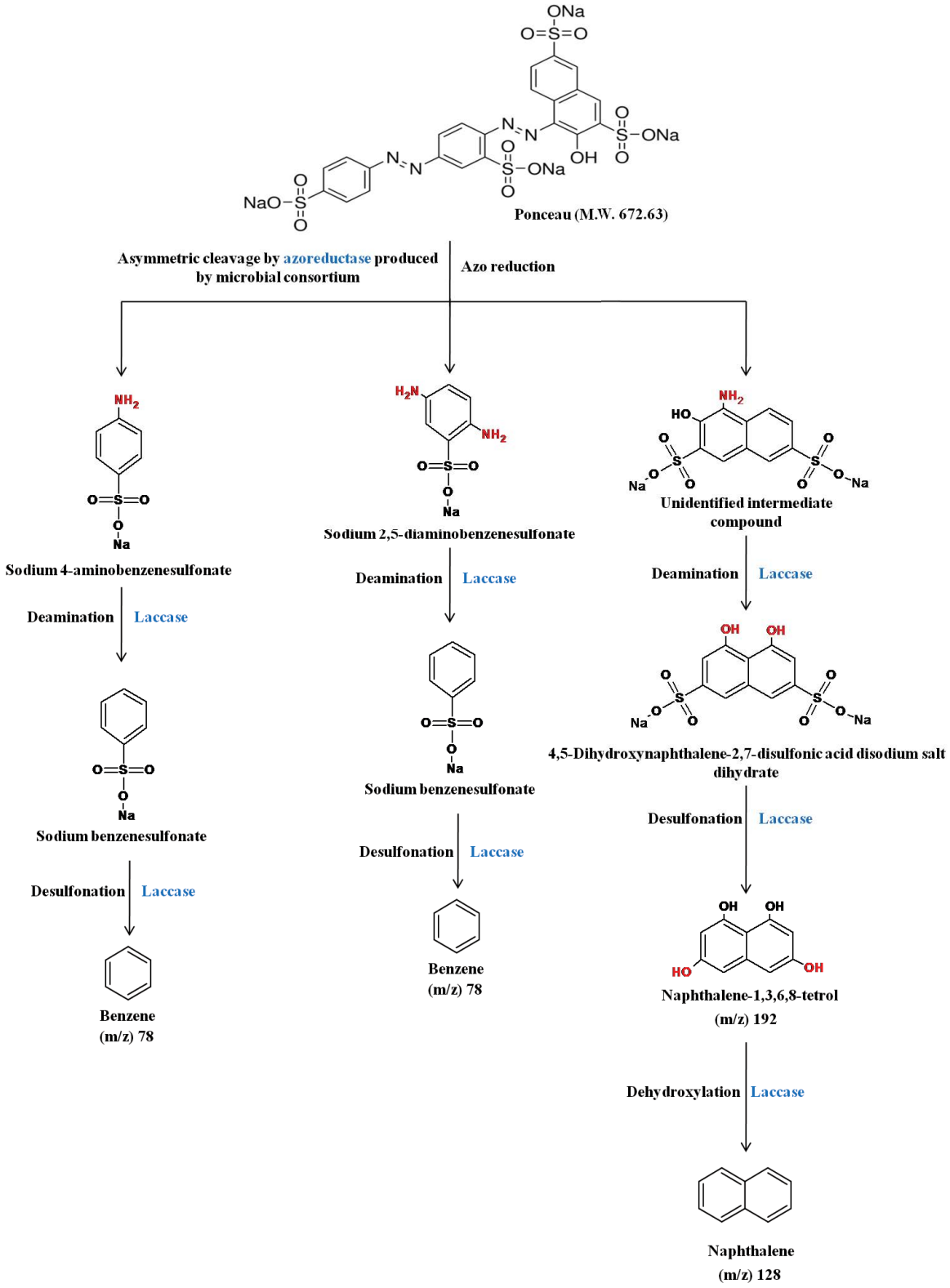


Fig. 12. Proposed pathways for the dye Ponceau degradation by immobilized consortium PS-PAAT.

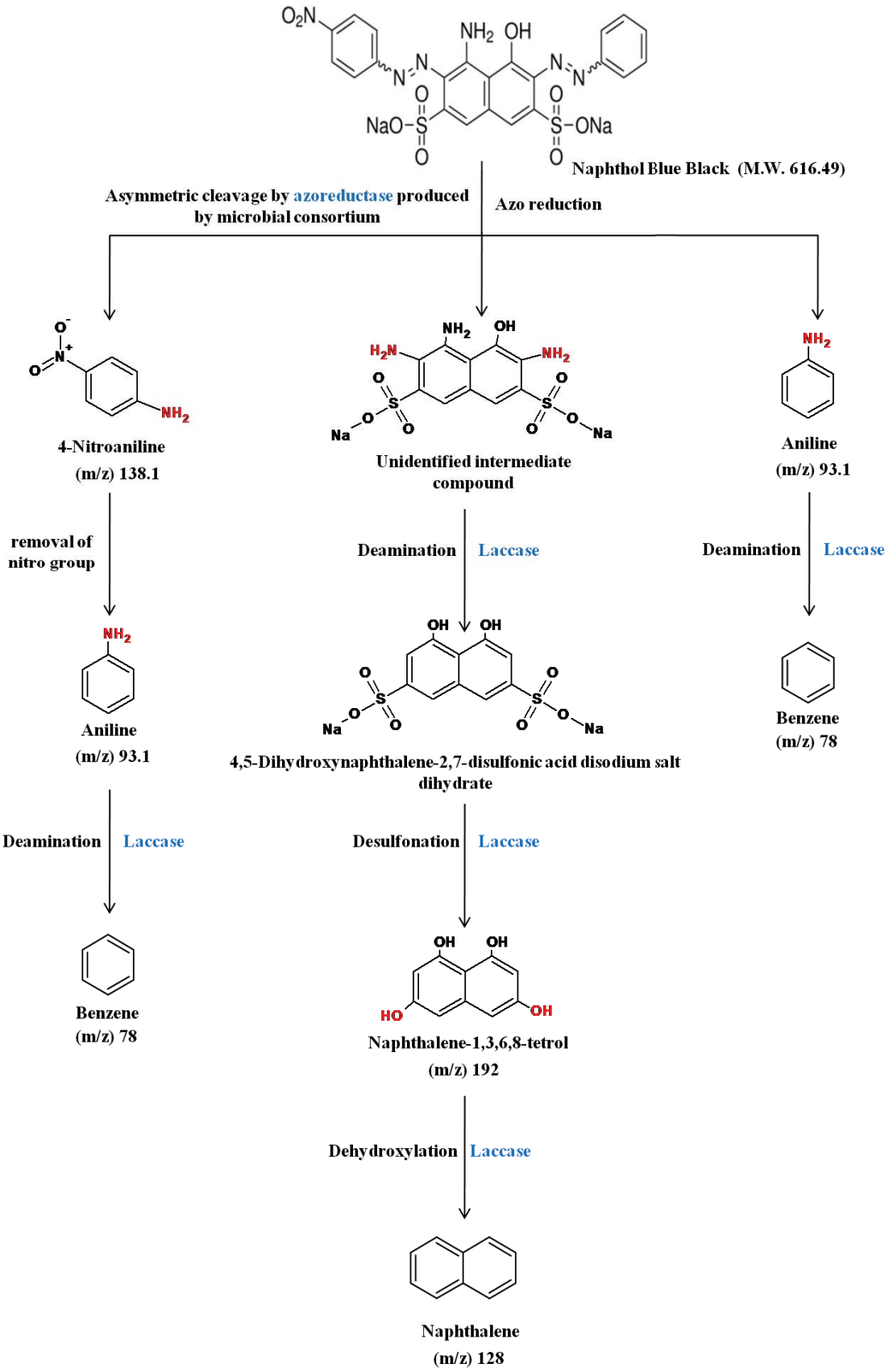


Fig. 13. Proposed pathways for the dye Naphthol Blue Black degradation by immobilized consortium PS-PAAT.

Table 4
Phytotoxicity studies of azo dyes and its metabolites formed after decolorization by immobilized consortium PS-PAAT

Parameters studied	Distilled water	500 ppm		500 ppm		500 ppm		500 ppm	
		AB 113	Metabolites	CR	Metabolites	DB 106	Metabolites	NBB	Metabolites
Vigna mungo									
Germination (%)	100	40	100	40	100	50	90	50	100
Plumule (cm)	11.6 ± 0.9	6.9 ± 1.3	10.8 ± 0.4	6.2 ± 0.3	11.1 ± 0.9	5.3 ± 1.2	9.2 ± 1.4	7.6 ± 0.8	10.1 ± 0.6
Radical (cm)	6.8 ± 1.2	4.2 ± 1.2	6.5 ± 1.3	3.7 ± 1.3	5.6 ± 1.5	3.2 ± 0.9	4.8 ± 0.8	5.1 ± 0.9	6.6 ± 0.6
Sesamum indicum									
Germination (%)	100	50	90	30	90	50	90	50	100
Plumule (cm)	10.3 ± 0.8	3.3 ± 0.2	7.2 ± 0.4	4.8 ± 0.2	9.9 ± 0.3	3.8 ± 0.3	10.1 ± 0.6	4.5 ± 0.4	8.8 ± 0.2
Radical (cm)	5.6 ± 1.7	1.8 ± 0.4	4.3 ±0.3	1.9 ± 0.3	5.1 ± 1.1	2.8 ± 1.2	4.1 ± 0.2	3.9 ± 1.3	5.3 ± 0.9
Parameters studied	Distilled water	500 ppm		500 ppm		500 ppm		500 ppm	
		P	Metabolites	RB 5	Metabolites	T	Metabolites	TB	Metabolites
Vigna mungo									
Germination (%)	100	60	100	60	90	50	80	30	100
Plumule (cm)	11.6 ± 0.9	8.7 ± 0.4	10.3 ± 0.3	6.8 ± 0.4	10.6 ± 1.3	6.2 ± 0.2	9.9 ± 1.2	8.8 ± 1.2	10.2 ± 1.3
Radical (cm)	6.8 ± 0.2	2.3 ± 0.1	5.7 ± 0.4	2.9 ± 0.2	6.3 ± 0.1	3.4 ± 0.3	6.3 ± 0.3	2.8 ± 0.2	6.1 ± 0.6
Sesamum indicum									
Germination (%)	100	40	100	30	90	40	80	40	100
Plumule (cm)	10.3 ± 0.8	6.7 ± 0.5	8.9 ± 0.3	5.8 ± 0.4	9.2 ± 0.5	4.9 ± 0.2	7.5 ± 0.3	6.5 ± 0.4	9.8 ± 0.3
Radical (cm)	5.6 ± 1.7	1.8 ± 0.4	4.6 ± 0.2	1.3 ± 0.1	4.9 ± 0.4	2.2 ± 0.5	3.9 ± 0.2	2.2 ± 0.2	5.4 ± 0.3

Values of the mean of six experiments standard error mean (SEM) (±), seeds germinated in azo dyes are significantly different from the seeds germinated in distilled water (control) and degraded metabolites (treated) at $p < 0.05$ by one-way ANOVA with Tukey–Kramer comparison test.

Table 5
Microbial toxicity assay of azo dyes and its metabolites formed after decolorization by immobilized consortium PS-PAAT

Parameters	Test organism	150 ppm		150 ppm		150 ppm		150 ppm	
		Acid blue 113	Metabolite	Congo red	Metabolite	Disperse blue	Metabolite	Naphthol Blue Black	Metabolite
Zone of inhibition (cm)	*Azotobacter vinelandii* MTCC 2460	1.2 ± 0.06	NI	0.8 ± 1.1	NI	0.6 ± 0.4	NI	0.9 ± 0.4	NI
	Pseudomonas putida MTCC 2476	1.2 ± 0.09	NI	0.8 ± 0.08	NI	0.6 ± 0.03	NI	0.9 ± 0.9	NI
Parameters	Test organism	150 ppm		150 ppm		150 ppm		150 ppm	
		Ponceau S	Metabolite	Reactive Black 5	Metabolite	Tartrazine	Metabolite	Trypan Blue	Metabolite
Zone of inhibition (cm)	*Azotobacter vinelandii* MTCC 2460	0.6 ± 1	NI	0.9 ± 0.3	NI	1.3 ± 0.3	NI	1.4 ± 0.6	NI
	Pseudomonas putida MTCC 2476	0.6 ± 0.09	NI	0.9 ± 0.5	NI	1.3 ± 0.6	NI	1.4 ± 0.6	NI

Values represent the means of the zone of inhibition (cm) from the three independent experiments, whereas (NI) indicates that there is no zone of inhibition observed in dye degraded metabolites.

substrate specificity (azo dye) and capable of decolorizing azo dyes at higher concentrations and required much less incubation time relative to the free suspended cell under optimized conditions. Moreover, immobilized bacterial-fungal consortia have gained certain advantages. One important advantage of the biodegradation system is the production of higher levels of oxidoreductive enzymes involved in the biodegradation of the azo dyes. The calcium-alginate fibers showed better stability during storage and operation than the free cell for each repeated batch degradation. The results indicate that the alginate-encapsulated consortium could be used as an effective system for environmentally safe biotransformation of the various textile dyes assayed in this work.

Acknowledgments

The authors acknowledged the management of SASTRA Deemed to be University, and Department of Chemistry and Biosciences, Srinivasa Ramanujan Centre for providing facility to carry out the research works.

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