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Biotransformation of mixture of dyes by enriched bacterial consortium ASD

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ABSTRACT

Dye effluents released from textile industries has gradually entrenched the roots of peril to majority of the water bodies. Considering the alarming need of innovative bioremediation technologies, the present study aims to evaluate the biodegradation potential of a bacterial consortium ASD for mineralization of a mixture of structurally different dyes. Consortium ASD, developed from dye contaminated soil from Vatva industrial estate, constitutes of four bacterial strains viz. *Pseudomonas* sp., *Brevibacillus* sp. and two strains of *Stenotrophomonas* sp. The intense metabolic activity of these strains together, led to 94% ADMI removal (American dye Manufacturers Institute) of 100 mg l^{-1} of a mixture of dyes in the presence of starch (0.1%, w/v) and proteose peptone (0.4%, w/v) within 24 h at 37˚C under static (microaerophilic) condition and could tolerate up to 400 mg l^{-1} of dye mixture. Consortium was also able to reduce total organic carbon and chemical oxygen demand by 85 and 78% within 24 h. Significant level of various oxido-reductive enzymes observed during decolourization by the consortium, confirms their involvement in the degradation process. Biotransformation of the mixture of dyes to less toxic byproducts was confirmed using Fourier transformed infrared spectroscopy, NMR, GC–MS analysis, and phytotoxicity test. Results showed a suitable application of consortium ASD for the biological processing of dye containing wastewaters.

Keywords: Bacterial consortium; Azo dye mixture; Decolourization; Biodegradation; Detoxification; Simulated microcosm

1. Introduction

Wastewater disposal is a major issue arising in the emerging industrial sector. Effluent discharges originating from textile mills, paper and printing industries, paints and color factories cause severe water pollution due to its toxicity and color contents. The occurrence of dyes in wastewater mainly contributes to the color of the industrial effluent. The loss of dyestuff in wastewater stream is as a result of an inefficient dyeing process in textile industries. The sulfonic and azo groups confer xenobiotic nature to the dyes, making them resistant to biological degradation [1].

The majority of the research focuses on developing effective treatment of dye containing wastewater, but no single solution is satisfactory for remediation of the broad spectrum of textile dye wastewater [2]. Several physicochemical decolourization techniques were

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accepted by the textile industries [3,4] but since past few decades, systems based on biological processes have gained immense attention [5,6]. Degradation of synthetic dyes by microbial consortium has been reported advantageous over the use of pure cultures [7–9]. Reports suggest it is always beneficial to use consortium, a suitable combination of efficient strains, for the bioremediation of waste containing variety of dyes as it provides a rich metabolic network. Additionally, consortium can withstand high stress of pollutants, due to the close interaction among the individual strains [8,9]. Study by Nasseri et al. [10] found improved biodegradation in a consortium-augmented bioreactor compared to the isolated microbe. It has been verified that indigenous microbial biomass is significantly better at biodegradation than the one commercially obtained [11]. Hence, there is a need to promote innovative biological decolourization processes leading to effective cleanup of azo dyes [12].

The objective of the present study was to develop a novel consortium named ASD by enrichment culture technique from contaminated soil of an industrial estate. Various physicochemical conditions have been optimized for effective and affordable decolourization of mixture of dyes. The end products formed after dye decolourization have been analyzed by Fourier transformed infrared spectroscopy (FTIR), NMR, and GC–MS to ensure complete dye degradation. The study was also focused to assess the toxicity of mixture of dyes before and after decolourization by consortium ASD. Soil microcosm study would impersonate the actual process performance under field condition.

2. Materials and methods

2.1. Dyes and chemicals

The dyes used in the study were of industrial grade and a generous gift from Vidhi Textile Industry, Vatva GIDC (Gujarat Industrial Development Corporation), Ahmedabad, India. The dyes (Direct yellow 11, Reactive Violet 5, Reactive Red 195, Reactive Orange 122, Reactive blue 5, Acid black 194, Direct Red 81, Reactive Blue 25, Orange M2R, and G-yellow MR) are listed in Supplementary Table S1. All chemicals and molecular biology reagents were of analytical grade such as Qualigen (Mumbai, India), Merck (Darmstadt Hesse, Germany), and Sigma-Aldrich (Steinheim, Germany).

2.2. Media

Bushnell and Haas medium ($MgSO_4$, 0.2 g l^{-1}; K_2HPO_4, 1.0 g l^{-1}; $CaCl_2$, 0.02 g l^{-1}; $FeCl_3$, 0.05 g l^{-1}; NH_4NO_3, 1.0 g l^{-1}; pH 7.2) supplemented with glucose (0.1%, w/v), yeast extract (0.05%, w/v), and 100 mg $l^{-1}$of dye mixture (stock of a mixture of dyes was prepared by adding an equal quantity of 10 individual dye to make a final concentration of 50,000 mg l^{-1}). For convenience, above modified BHM will be referred as MBHM.

2.3. Development and identification of bacterial consortium ASD

Soil samples were collected from contaminated sites near textile dyeing industries at Vatva GIDC, Gujarat and 10% (v/v) of soil suspension (10 g l^{-1}) was added to 100 ml MBHM in 250 ml Erlenmeyer flasks, incubated at 37˚C on orbital shaker (150 rpm) as well as under static conditions at 37˚C. Among developed consortia, consortium ASD having maximum decolourization efficiency with respect to time was selected for further studies. Repeated transfers were carried out in fresh MBHM till stable dye decolourizing consortium (ASD) was obtained showing consistent growth and decolourization at every successive transfer. In addition, increasing the number of transfers into fresh media ensured the less possibility of unculturable bacteria in the consortium.

The appropriate dilutions of consortium ASD were spread onto Luria Bertani agar and Reasoner's Agar (R_2A agar) and incubated at various temperatures for different time intervals. Based on distinct morphology, different bacterial colonies were isolated. Genomic DNA was extracted from individual bacterial isolate as per Ausubel et al. [13]. The 16S rRNA gene was amplified by PCR employing the universal primers, 8F and 1492R as described in Desai and Madamwar [14]. Sequencing was done using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences from each isolates were compared to the existing database of NCBI. Phylogenetic tree was constructed by the neighbor joining method using MEGA 5.2 version.

2.4. Decolourization studies

All experiments were conducted in 250 ml Erlenmeyer flask containing 100 ml MBHM inoculated with 10% (v/v) bacterial consortium ASD. Uninoculated medium under similar conditions served as abiotic control. Supernatant was obtained by centrifuging sample at 7,000 × *g* (Kubota 6500, Japan) for 10 min which was analyzed spectrophotometrically (Specord-160 UV/visible, Analytik Jena AG, Germany). The ADMI value of a mixture of dyes was determined

using the "Adamse Nickerson chromatic value formula" [15]. The percent ADMI removal was calculated based on initial and final ADMI values obtained against uninoculated control [16,17]. For all the rest of experiments, percent ADMI removal will be referred as percent decolourization. Cell density was monitored by checking the absorbance at 600 nm against distilled water.

2.4.1. Optimization of nutritional and physicochemical parameters

To study the effect of nutritional parameters for maximum decolourization of the dye mixture by bacterial consortium ASD, MBHM with varying carbon sources and nitrogen sources at different concentrations ranging from 0.1–0.5% (w/v) were optimized. Effect of salinity on decolourization was investigated in the presence of varying concentration of NaCl (5–25%, w/v).

Various physicochemical parameters such as the optimum temperature (20–45˚C), pH (2.5–8.5), initial dye concentration (100–500 mg l^{-1}), and inoculum size (10–50%, v/v) were monitored for decolourization by the consortium. The sets were incubated at 37˚C under static condition and samples were aseptically withdrawn at regular time intervals of 12 h until 48 h. All experiments were done in triplicates.

2.4.2. Decolourization with repeated addition of dye aliquots

The reusability of microorganisms was checked by the ability of bacterial consortium ASD to decolorize repeated addition of dyes at 37˚C under static condition. Mixture of dyes at a concentration of 100 mg l^{-1} was spiked at regular interval every 24 h up to 144 h.

2.4.3. Decolourization of mixture of dyes by individual culture

The decolourization of a mixture of dyes (100 mg l^{-1}) was evaluated using the individual bacterial isolates in MBHM along with starch (0.1%, w/v) and proteose peptone (0.4%, w/v) at 37˚C under static condition. Aliquots were removed at regular intervals and analyzed for percent ADMI removal as a measure of percent decolourization.

2.5. Enzymatic studies

2.5.1. Preparation of cell free enzyme extract

Consortium ASD and its individual constituent strains were grown in dye containing broth at 37˚C for 24 h, followed by centrifugation at 6,000 × *g* for 20 min to harvest the actively growing cells. The cell pellet of individual cultures and consortium was washed twice with 50 mM potassium phosphate buffer (pH 7.4) and sonicated (Sonics Vibracell ultrasonic processor, USA) separately by giving seven strokes, each of 40 amplitude for 30 s with 2 min of time interval between two strokes to disrupt cell wall. This extract was centrifuged at 10,000 × *g*, 4˚C, for 20 min and the supernatant was used as a crude enzyme for further assay.

2.5.2. Enzyme activities

Lignin peroxidase activity was monitored by the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 ml. The assay mixture contains 250 mM tartaric acid, 100 mM n-propanol, 10 mM H_2O_2, and 150 µl cell free extract Telke et al. [18]. Laccase activity was determined by the oxidation of ABTS at 420 nm following the method of Hatvani and Mecs [19] with little modifications. The reaction mixture of 2.0 ml contains 0.3 M ABTS in 0.1 M acetate buffer (pH 4.9) along with 150 µl cell free extract. Tyrosinase activity was determined at 410 nm for the oxidation of catechol. The reaction mixture of 2.0 ml contains 5 mM catechol in 100 mM potassium buffer (pH 7.4) along with 150 µl cell free extract. All sets for enzyme activity analysis were studied under ambient conditions, where reference blanks contained all component except crude enzyme. All enzyme assays were conducted in triplicate and the average rates were calculated to represent the enzyme activity. One unit of enzyme activity was defined as a change in absorbance Unit min^{-1} mg $protein^{-1}$ of the enzyme.

Azo-reductase activity was measured by monitoring the decrease in concentration of Methyl Red at 440 nm. The reaction mixture of 3 ml consisting of phosphate buffer (100 mM, pH 7.0), NADH (1 mM), 150 µM Methyl Orange, and 100 µl of enzyme containing 38 µg of protein. The reaction was initiated by adding NADH. NADH-DCIP reductase activity was assayed with few modification by the method reported by Bhosale et al. [20]. Reaction mixture of 5.0 ml containing 50 µM DCIP (substrate), 50 mM potassium buffer (pH 7.4) along with 150 µl crude enzyme (cell free extract). The reduction of DCIP was measured at 595 nm by adding 50 µM NADH. Flavin oxidoreductase was measured by the procedure of Fontecave et al. [21] with some modification. Crude enzyme i.e. cell free extract was added to a reaction mixture consisting of 100 µM of Tris-HCl (pH 7.5), 25 µmol of NADPH, and 0.003 unit riboflavin in a 1 ml system. The decrease in absorbance at 340 nm

was analyzed spectrophotometrically. One unit of enzyme activity was defined as a microgram of riboflavin reduced per min per mg of protein. Enzyme assays were performed in triplicates. One-way ANOVA with Tukey–Kramer comparison test was implemented for statistical analysis.

2.6. Analysis of degradation products

To understand the degree of degradation of a mixture of dyes, reduction in the chemical oxygen demand (COD) [15] was determined after 24 h incubation with consortium ASD. In addition, the total organic carbon (TOC) of the culture before and after decolourization was also measured by using a TOC analyzer (Shimadzu 5000A, Japan).

The end products produced during biodegradation under microaerophilic conditions were analyzed using FTIR, NMR, and GC–MS as described by Jain et al. [22]. FTIR analysis was carried out using Perkin Elmer, spectrum GX spectrophotometer in the mid infrared region of 400–4,000 cm^{-1} to 16-scan speed. ^{1}H Nuclear magnetic resonance (^{1}H NMR) studied using Bruker ^{13}C NMR-400 MHz. Gas chromatography-Mass Spectrometry (GC–MS) analysis of metabolites was performed using Auto-system XL (Perkin Elmer, USA).

2.7. Toxicity study on plant

The byproducts obtained after degradation of the dye mixture were examined for phytotoxicity against *Triticum aestivum* var. GW173 and *Vigna radiate* var. Meha. Seeds were collected from the local market. The seeds were presoaked for 4 h in the solution of mixture of dyes and then germinated on 1% agar plates under dark condition. Seeds soaked in distilled water were considered as experimental controls. After 7 d, % germination, length of plumule (shoot) and radicle (root) of seeds was measured. Seeds with radicle (>1 mm) were considered germinated [23]. All experiments were carried out in triplicates.

2.8. Efficiency of consortium ASD in dye amended soil

Simulated microcosms were prepared as mentioned by Pathak et al. [24]. The soil from institutional garden was sieved (<2 mm) and characterized for its physicochemical properties (native moisture content 35 ± 3%, pH 6.5). Each microcosm was prepared in 200 ml glass beakers containing 50 g of soil. The cells from consortium were harvested in late log phase by centrifugation at 7,000 × *g* for 10 min, washed thrice with sterile deionized water, and resuspended in the same to get 1.0 O.D. at 600 nm. The different permutation combination of sets was selected for study in triplicates bearing the details as given in Table 1. After inoculation (20% v/w), the soil was thoroughly homogenized and incubated at 37˚C. The moisture content was maintained at 40%. Percentage of dye decolourization and CFU of culturable bacteria were monitored after 24 h incubation for next two days.

3. Results and discussion

3.1. Development of dye decolourizing bacterial consortium

Continuous release of dyestuff laden wastewater from the various industries of Vatva, GIDC contaminates the nearby area and water bodies. The persistent pollution load causes microbial community within the polluted site to assimilate and adapt to the organic pollutants. Soil samples from such contaminated site were enriched to develop an efficient dye decolourizing bacterial consortium.

Decolourization by two or more bacterial isolates has been reported to occur only when carbon and nitrogen source is available in the growth medium. Hence, glucose and yeast extract were employed as co-substrates during enrichment process, which seems to be obligatory for the dye decolorizing bacteria to show its effectiveness [25,26]. Among the various consortia developed, consortium ASD was the most efficient azo-dye decolourizing consortium. More than 100 repeated transfers of the consortium verify that the uncultivable portion might have gradually become extinct. Four diverse bacterial species were acquired from the consortium ASD, assigned to the genus namely; two strains of *Stenotrophomonas* sp. (KF185095, KF185098), *Pseudomonas* sp. (KF185096) and *Brevibacillus* sp. (KF185097) based on 16S rRNA sequence analysis data (Fig. 1).

3.2. Decolourization ability of consortium ASD under different physicochemical parameters

Decolourization studies revealed that consortium ASD can decolourize up to 100 mg l^{-1} of a mixture of dyes (≥74%) under static condition. While under shaking condition the consortium showed reduced decolourization of 38% for a synthetic mixture of dyes (Fig. 2). This might be due to the fact that bacterial decolourization is often an enzymatic reaction which is inhibited in the presence of oxygen due to the competition between azo group and oxygen as an electron receptor [27]. Similarly, Patel et al. [7] reported that decolourization of Acid Maroon was 94% under static condition, whereas 63% was obtained under shaking condition in 20 h by consortium EDPA.

Table 1
Simulated microcosm study measuring the ability of consortium ASD to degrade mixture of dyes in soil environment

No.	Experimental sets	Experimental parameter	Results
1	Set A	Sterile soil amended with mixture of dyes, to determine abiotic loss of dye	Dye decolourization was not observed (~2.1%)
2	Set B	Sterile soil amended with mixture of dyes and inoculated with consortium ASD to determine dye degradation ability of consortium ASD in absence of indigenous microflora	Consortium ASD could decolourize 89.9% of mixture of dyes
3	Set C	Nonsterile soil amended with dye mixture to evaluate the intrinsic ability of soil to biodegrade the dye	Only 6% decolourization was observed by the indigenous microflora
4	Set D	Nonsterile soil amended with mixture of dyes and inoculated with consortium ASD to check whether the strain accelerated the dye degradation while competing with indigenous microorganisms	Consortium ASD and indigenous microorganisms showed 90.2% dye decolourization
5	Set E	Sterile soil (without dye) inoculated with consortium ASD to determine the ability of consortium ASD to grow on the soil nutrients and colonize there in	Growth of consortium ASD was observed in soil in absence of mixture of dyes

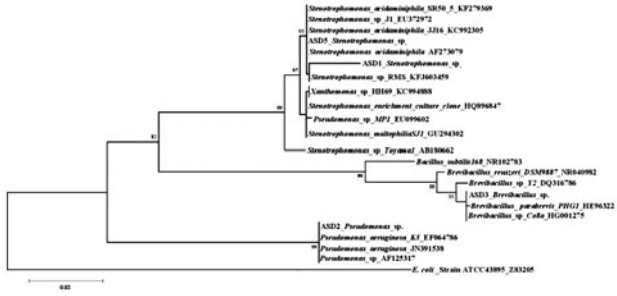

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence showing relationship between all the isolates present in consortium ASD. The sequence has been retrieved from NCBI database and the tree was constructed using neighbor joining method in MEGA 5.2 software. Numbers at nodes indicate percentage bootstrap value.

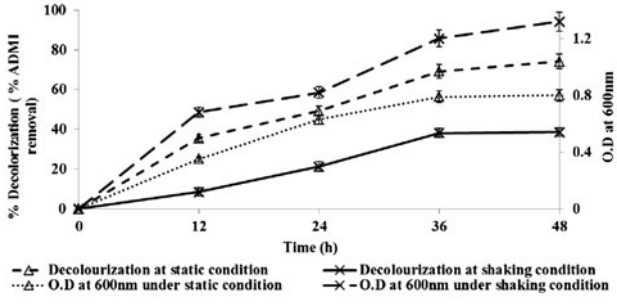

Fig. 2. Decolourization of mixture of dyes at static and agitated condition by consortium ASD and its growth at respective condition.

3.2.1. Effect of carbon sources on decolourization

The decolourization of consortium ASD in the presence of an additional carbon source was examined to obtain efficient and faster decolourization. The consortium ASD showed moderate decolourization in the presence of glucose, sucrose, fructose, and maltose up to 73, 68, 59, 45%, respectively at 0.1% (w/v) concentration. Starch was the most appropriate carbon source giving 81% decolourization of a mixture of dyes within 48 h (Fig. 3(a)). Upon optimizing the concentration of starch (0.1–0.5%, w/v) in the medium, maximum decolourization (82%) was observed at 0.1% (w/v) concentration in 48 h (Fig. 3(b)). Further increase in concentration caused no significant change in decolourization. Moosvi et al. [28] also used starch in place of glucose as an alternative co-substrate for the decolourization of nine different textile dyes by consortium JW-2. Using starch for dye decolourization being economically feasible, this system can be applied for the decolorization of dye containing industrial effluents relative to existing physicochemical methods.

3.2.2. Effect of nitrogen sources on decolourization

The nitrogen content of the medium has a marked effect on all parameters investigated. Fig. 3(c) clearly suggests higher decolorization of 85% in the case of proteose peptone compared to yeast extract, tryptone, beef extract, urea, and ammonium nitrate with 72, 63, 57, 54, and 41%, respectively within 48 h. Nigam et al. [25] used proteose peptone in their decolourization study other than yeast extract as a nitrogen source in

the medium with BHB and glucose and it exhibited good decolourizing ability of 90%. Organic nitrogen sources are reported to regenerate NADH, which acts as an electron donor for the reduction of azo dyes by microorganisms. Hu [29] has reported the growth-related decolourization to be directly associated with the concentration of yeast extract and at lower concentrations of yeast extract, growth reduced resulting in lesser color removal. Furthermore, different concentration of proteose peptone ranging from 0.1 to 0.5% (w/v) was tested and it revealed that increase in concentration of proteose peptone caused a significant increase in dye decolourization i.e. 90% in 48 h with 0.4% (w/v) of N content (Fig. 3(d)). But a further increase in concentration made no changes in percentage decolourization.

3.2.3. Effect of high salt concentration on decolourization of mixture of dyes

The spent dye bath effluents contain very high concentrations of salts and henceforth decolourization by biomass was assessed at high salt concentration.

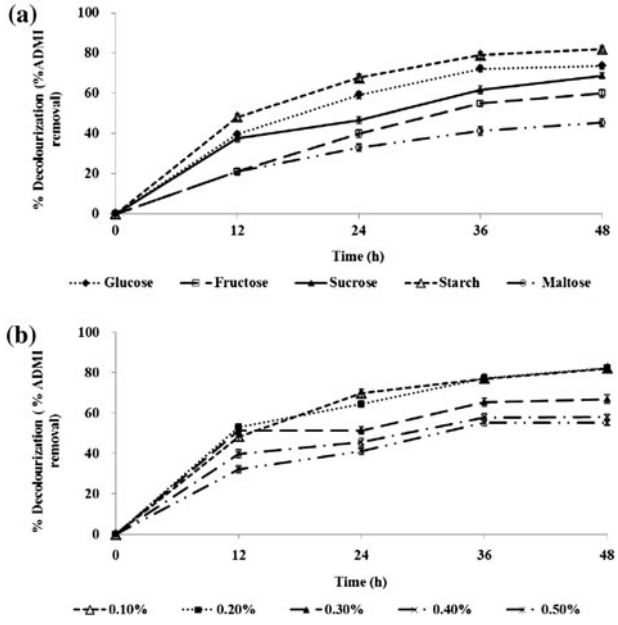

Fig. 3. Optimization of nutritional parameters for decolourization of mixture of dyes by consortium ASD: (a) effect of different carbon sources (0.1%) on dye decolourization along with YE (0.1%), (b) effect of increasing concentration of starch on decolourization, (c) decolourization of the mixture of dyes by consortium ASD using different organic and inorganic nitrogen source (0.1%) in BHM, (d) effect of increasing concentration of proteose peptone on decolourization, and (e) effect of varying salt concentration on decolourization of dye mixture (100 mg l^{-1}).

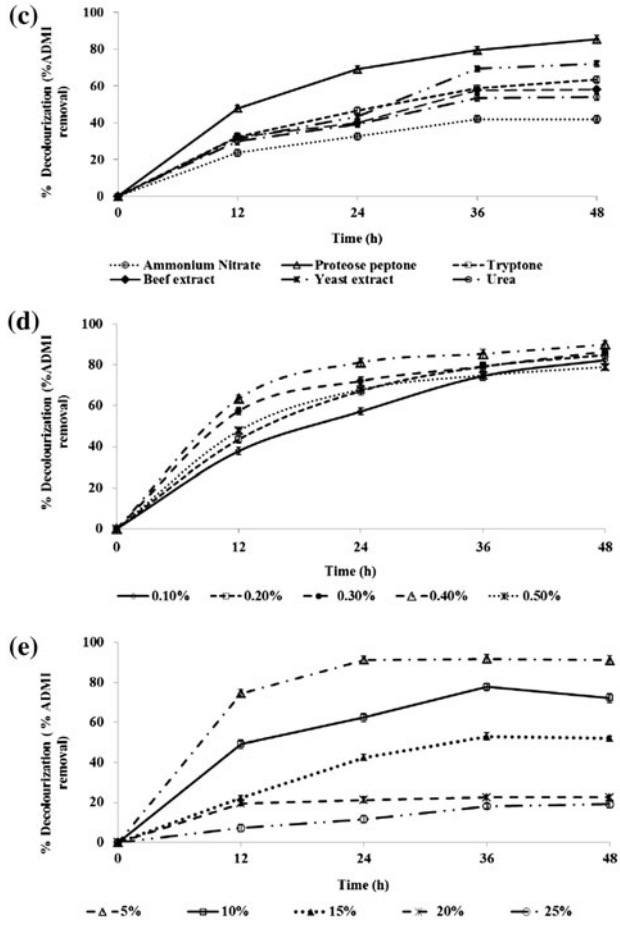

Fig. 3. (*Continued*).

The microaerophilic acclimatized biomass retained its efficiency of decolourization (75%) up to 6% w/v salt concentration within 24 h (Fig. 3(e)). However, the inhibitory effect of salinity becomes evident at higher salt concentrations as depicted by plot corresponding to 8% w/v salt (≥50% decolourization) concentrations. Conversely, Palani et al. [30] have reported the ability of *Bacillus* sp., *Enterobacter* sp., *Pseudomonas* sp., and *Alcaligenes* sp. to withstand a higher sodium chloride concentration up to 30 g l^{-1} without losing their decolourizing ability.

3.2.4. Effect of inoculum size on decolourization

To estimate the optimum inoculum size of consortium ASD, for 100 mg l^{-1} dye concentration, different inoculum sizes were used. The results indicate that dye removal increases with increase in inoculum concentration but beyond 20% (v/v) inoculum size, rate of increase in decolourization was not very significant (Fig. S1(a)). A similar pattern was observed by Sani and Banerjee [31] where there was no proportionate

increase in the decolourization with an increase in inoculum size of *Kurthia* sp. for the treatment of the textile effluent. With 20% inoculum size, maximum decolourization of 91% within 24 h of incubation was observed while in case of 30 and 40% inoculum size, decolourization was 75 and 67%, respectively. This observation was comparable to a report where 100% decolourization of Malachite Green (50 mg l^{-1}) was observed with 10% inoculum size by *Kocuria rosea* MTCC1532 [32].

3.2.5. Effect of pH and temperature on dye decolourization

The pH tolerance is an important consideration for industrial applications because processes using reactive azo dyes are usually performed under alkaline conditions [33]. The bacterial consortium could decolourize more than 90% of the dye mixture by incubating up to 24 h within a range of pH 6.0–7.0 with maximum decolourization of 93% at pH 6.5 (Fig. S1(b)). ASD decolourized azo dyes in a wide range of pH which can be considered as a desirable characteristic in contrast to the common individual decolourizing bacteria that have a narrow pH range [34–36]. At lower pH, the H^+ ions compete effectively with dye cations, causing a decrease in color removal efficiency. Furthermore, at high pH, the surface of the biomass gets negatively charged, which enhances the positively charged dye cations through electrostatic force of attraction [37]. Our observations indicate that the consortium can treat basic dyeing wastewater at normal operational pH and decrease the cost of acidification.

The mesophilic temperature range is commonly used for decolourization studies as maintaining high temperature would not be economical and dye degradation is slow within the psychrophilic range. Fig. S1 (c) shows that the decolourization rate increases with increasing temperature from 20 to 37˚C. The maximum decolourization of 94% was detected at 37˚C within 24 h. At 45˚C, the decolourization ability of the consortium was sharply reduced to 46%. This might have occurred due to adverse effects of high temperature on the enzymatic activities of the microbial consortium. The present study was in concurrence with those reported by Wang et al. [38] for the decolorization of Reactive Black 5 by a newly isolated bacterium *Bacillus* sp. YZU1 within 24 h.

3.2.6. Effect of various dye concentration

The higher concentration of azo dye inhibits nucleic acid biosynthesis and cell growth [34], so the effect of dye concentration on growth of organisms is an important parameter to be considered for its field application. Our results revealed that the rate and extent of decolourization were affected by the additions of increasing concentration of dye ranging from 100 to 500 mg l^{-1}. The dye concentration of 100 mg l^{-1} was decolorized up to 94% within 24 h, which consecutively decreased to 81, 72, 35, and 17% in case of 200, 300, 400, and 500 mg l^{-1}, respectively, of dye concentration (Fig. 4(a)). Similar observation has been reported for decolourization of triphenylmethane dyes by *Kurthia* sp. [31]. Khehra et al. [39] suggested that the decrease in decolourization efficiency might be due to the toxic effect of dyes. Initial concentration provides an important driving force to overcome all mass transfer resistance of the dye between the aqueous and solid phases [32].

3.2.7. Decolourization with repeated addition of dye aliquots

Repeated use of microorganisms is significant from a commercial point of view. This study was carried out to examine the ability of consortium ASD to decolourize dye mixture aliquots (100 mg l^{-1}) at static conditions on repeated additions. Upon 94% decolourization of first dye aliquot (Fig. 4(b)) within 24 h a

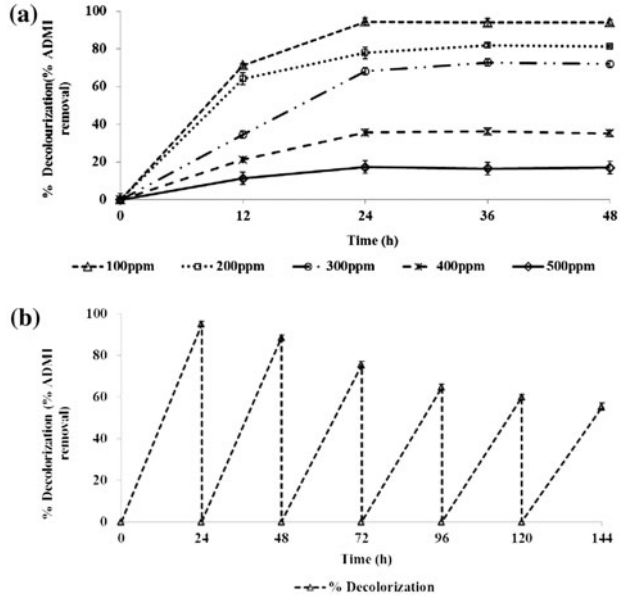

Fig. 4. Decolourization of dye mixture by consortium ASD: (a) different concentration of dye mixture to check the decolourization performance of consortium ASD and (b) repeated dye addition aliquots to check potentiality of consortium ASD.

second aliquot of dye was added, which was decolourized up to 86% within the next 24 h. The culture caused 78 and 64% decolourization up to third and fourth aliquot, which were subsequently added within 24 h. Further addition of dye decreased the decolourization performance with the increase in the incubation period. The eventual cessation of decolourization might be due to nutrient depletion, which is directly related to microbial growth and enzyme activity [40].

3.2.8. Decolourization of mixture of dyes by individual culture

Each bacterial culture from the consortium was tested individually for its ability to decolourize in liquid medium and all showed decolourization upon extended incubation (data not shown). Results indicated that strains of *Stenotrophomonas* sp. (KF185095) and *Pseudomonas* sp. (KF185096) were able to decolourize mixture of dyes up to 62 and 66% at 100 mg l^{-1} within 24 h. Whereas, 52% of decolourization was observed in case of *Stenotrophomonas* sp. (KF185098) within 24 h. However, decolourization was not significant (32%) when dye containing medium was inoculated with *Brevibacillus* sp. (KF185097), but presence of this strain might be playing a crucial role to withstand the high concentration of dye compounds. However, when all the four bacterial cultures were mixed and inoculated together in MBHM medium, complete decolourization of a mixture of dyes was observed within 24 h signifying a synergistic role of the bacterial species in decolourization [41].

3.3. Enzymatic assay

A major mechanism behind biodegradation of different recalcitrant compounds in microbial system is mainly due to the activity of the biotransformation enzymes [20]. In order to obtain additional insight into the decolourization mechanism, enzyme activities of lignin peroxidase, laccase, NADH-DCIP reductase, azoreductase, and riboflavin reductase were monitored over time. Enzyme activity was checked in consortium and its constituent strains. It was observed that cell supernatant exhibits negligible enzymatic activity as compare to cell free extract and henceforth, it was not taken into consideration. As shown in Table 2, significant level of oxidative enzyme activity of lignin peroxidase with 4.89 units and laccase with 1.46 units were observed over the period of decolourization by consortium ASD as compared to riboflavin reductase with 4.8 units, azoreductase with 3.8 units, NADH-DCIP reductase with 5.3 units after complete decolourization in 24 h. The enzymatic profile presumably indicates the involvement of oxidoreductive enzymes for the degradation of a mixture of dyes into simple metabolites by consortium ASD under microaerophilic condition. As expected, the increased decolourization rate by the consortium than the individual cultures might be due to the synergistic enzymatic actions of all the organisms in the consortium.

3.4. Determining the degree of mineralization by ASD via TOC and COD measurement

To evaluate the decolourization and biodegradation level of the azo dyes by consortium ASD, the percentage of mineralization (represented by TOC and COD removal ratio) was determined by measuring the initial and final organic content of dye in the sample. In the presence of consortium ASD, moderate TOC removal of 85% and COD reduction of 78% were observed after 24 h of incubation, indicating mineralization of dye mixture (Fig. S2). These results suggest that decolourization by consortium might be largely attributed by biodegradation rather than biosorption of dye mixture.

3.5. Biodegradation analysis

The biodegradation of a mixture of dyes was assessed using FTIR, NMR, and GC–MS. FTIR spectrum (400–4,000 cm^{-1}) of dye mixture and its biodegraded metabolites is shown in Fig. 5. FTIR spectrum showed different peaks at 3,217, 2,956, 1,668, and 1,455, and 1,543 cm^{-1} (Fig. 5(a)) corresponding to the presence of an alkyne (≡C–C–), carboxylic (–COOH), carbon double bond (–C=C–), and an asymmetric $–NO_2$ group, respectively. A characteristic peak at 1,189 and 1,378 cm^{-1} indicates the presence of S=O stretching, asymmetric in case of sulfonic acids that signifies the occurrence of sulfonated dye compounds such as Reactive Blue and Reactive Orange in the dye mixture [42]. Moreover, aromatic ring meta-substituted groups corresponds to 703 cm^{-1}, and a peak at 1,309 cm^{-1} corresponds to amine ($–NH_2$). In addition, the bands located within the range of 1,610–1,630 cm^{-1} contributes to azo linkages –N=N– on aromatic structures [43]. Concurrently, after complete decolorization, the absence of bands near 950–620 cm^{-1} within 24 h demonstrates loss of aromaticity or fission of benzene rings (Fig. 5(b)). The absence of peak near 1,610–1,630 cm^{-1} indicates disruption of azo bond [44], while a peak at 2,924 cm^{-1} represents asymmetric stretching of alkenes. It was observed that no new

Table 2
Oxido-reductive enzyme profile of consortium ASD and its constituent strains after 24 h incubation with 100 mg l^{-1} dye mixture

Enzymes	ASD1	ASD2	ASD3	ASD4	Consortium ASD (24 h)
Lignin peroxidise[a]	1.10 ± 0.003**	ND	2.1 ± 0.02**	0.92 ± 0.002**	4.89 ± 0.09**
Laccase[a]	0.51±0.002*	ND	0.43 ± 0.003*	0.12 ± 0.005*	1.46 ± 0.002*
Tyrosinase[a]	0.04 ± 0.01**	ND	0.03 ± 0.009**	0.01 ± 0.02**	0.08 ± 0.002**
Azoreductase[b]	0.20 ± 0.002**	1.90 ± 0.15***	ND	1.70 ± 0.007***	3.80 ± 1.60***
NADH-DCIP[c]	0.50 ± 0.02***	3.20 ± 0.09**	ND	1.10 ± 0.03***	5.3 ± 1.80***
Riboflavin reductase[d]	0.90 ± 0.11***	2.10 ± 0.35***	ND	1.80 ± 0.08***	4.80 ± 2.20***

[a]Enzyme unit/min mg protein.
[b]μM methyl red reduced/min mg protein.
[c]μg DCIP reduced/min mg protein.
[d]1 g of riboflavin reduced mg^{-1} min^{-1} protein, ND = not detected.
*Values are mean of three experiments ± standard deviation, significantly different from control at: $p < 0.05$.
**$p < 0.01$.
***$p < 0.001$ by one way ANOVA with Tukey–Kramer comparison test.

peaks appeared between 3,300–3,500 cm^{-1} and in the region between 1,340 and 1,250 cm^{-1} after complete mineralization (24 h) suggesting that the azo linkage could be transformed into N_2 or NH_3 or incorporated into the biomass [45,46].

The ^{1}HNMR spectrum of control and degraded samples are shown in Fig. S3. ^{1}HNMR spectra of a mixture of dyes showed several signals recorded in up and down fields (Fig. S3(a)). A signal recorded at ppm 4.88 indicates the presence of methoxy group while signals at 4.83 and 2.20 ppm corresponds to the presence of multiplet of six protons responsible for two protons each for olefinic, enolic, alcoholic, and aliphatic groups, respectively. In down field region, signals between 7.52 and 7.55 ppm depicts the presence of aromatic protons, while signals at 8.26 and 8.66 ppm are from naphthylic hydrogen from dye molecule [39]. The downshift signals of 6.9 and 7.2 ppm corresponds to benzene sulfonic moieties and signals recorded near 3–4 ppm confirmed the presence of halide group suggesting the presence of sulfonic dyes in the mixture. After complete degradation of the dye mixture, several signals in the range of 5.30–6.0 ppm appeared. These could correspond to unstable alkene compounds resulting from ring openings of different aromatic products (Fig. S3(b)). Moreover, only a few signals were remaining in the region of 1.0–3.0 ppm, which resulted due to lower molecular weight aliphatic hydrocarbons such as free $–CH_3$, etc., and no peaks corresponding to the aromatic protons were found. Thus, in addition to reduction of azo bond, ring fission was also observed.

GC–MS analysis was performed to get further confirmation on dye degradation. The identification of intermediary products was carried out based on their mass spectra, molecular weight, and chemical structure. According to our study within the initial 12 h of incubation, the azo bond might have asymmetrically cleaved that lead to formation of sodium 4-diazenyl-benzenesulfonate, Sodium 2-(3-diazenyl-4-hydroxyphenylsulfonyl) ethyl, and 4 nitrophenyl diazine with mass peak at 207, 331, and 151, respectively. The base peak at m/z 246, 404, 489, and 281 were identified as sodium 7-hydroxy naphthalene-2-sulfonate, sodium-4-acetamido-5hydroxy-naphthalene2,7-disulfonate, sulfate1-amino4-(3-amino-4-sulfo-phenylamino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid, and amino-phenyl sulfonyl) ethyl hydrogen sulfate, respectively (Table S2). During the later stage of incubation (24 h), reductive enzymes might have played an important role leading to the formation of low molecular weight compound such as benzene, naphthalene, and aniline with mass peak at 75, 128, and 93, respectively. Further benzene and naphthalene might have entered into the TCA cycle [47], while aniline entered in TCA cycle via ring fission of catechol leading to mineralization of dyes upon extended incubation.

3.6. Phytotoxicity studies

Despite the fact that untreated dyeing effluents cause serious environmental and health hazards, they are being disposed off in water bodies and this water is being used for agricultural purpose [48]. Use of untreated and treated dyeing effluents in the agriculture has a direct impact on fertility of soil. Concerning the phytotoxicity, the relative sensitivity of *Vigna radiata* and *Triticum asetivum* seeds toward the mixture of

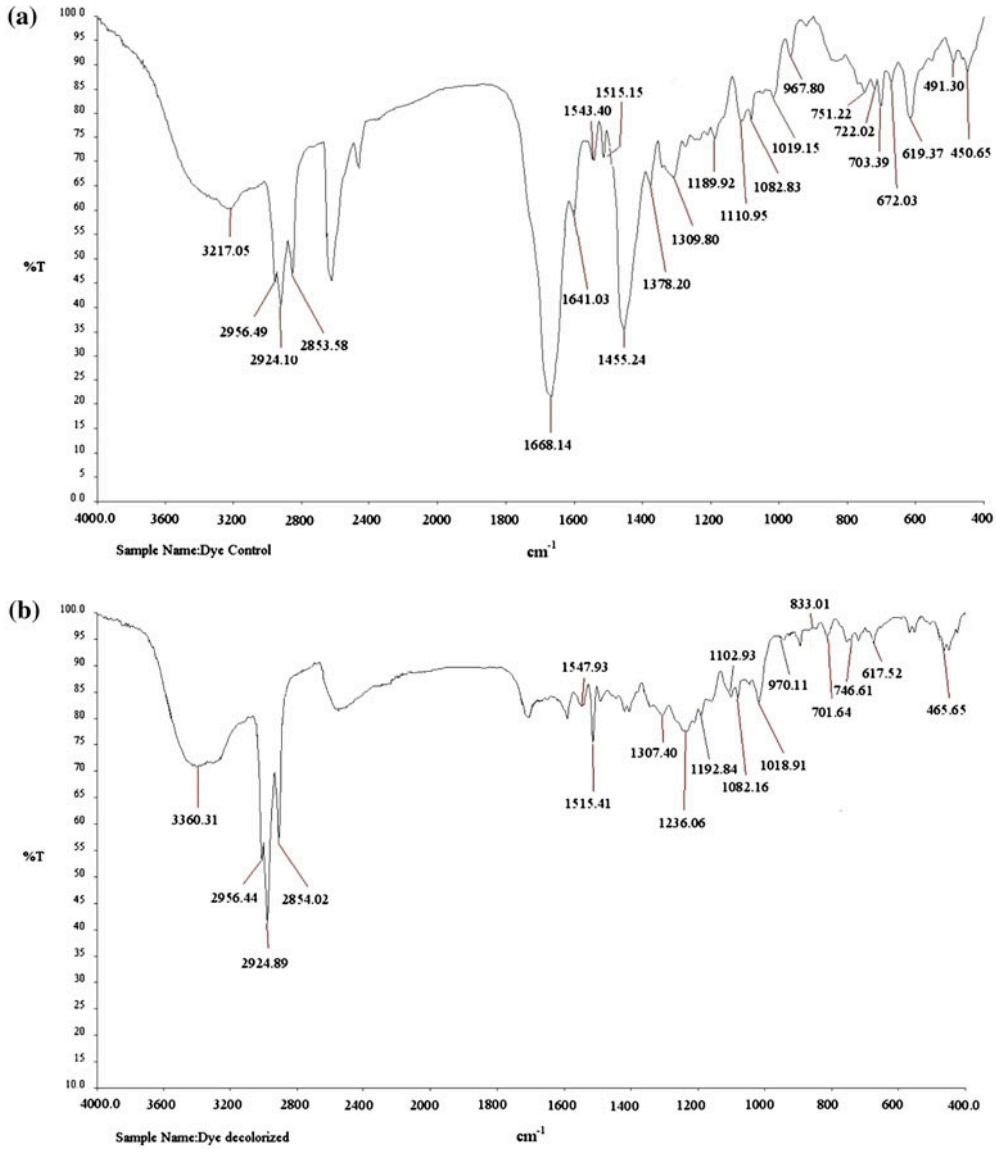

Fig. 5. FTIR spectrum of (a) dye mixture (100 mg l^{-1}) and (b) ethyl acetate extracted metabolites after complete decolourization (24 h) by consortium ASD at 37°C under static condition.

dyes and its degradation products by consortium ASD were studied. The germination of the seeds was quite low when treated with a mixture of dyes. In contrast, efficient shoot and root length was found when treated with a degraded product of dyes (Table 3). Our results indicate that the decolourization by the consortium confers nontoxic metabolites conferring better germination rate as well as significant root and shoot length of *Vigna radiata* when compared to germination by dye mixture where inhibition in all these parameters was observed. These results are in accordance with that reported by Parshetti et al. [32].

3.7. Simulated microcosm study

Simulated microcosm studies were conducted to find out the potential of consortium ASD to grow and survive in soil besides dye degradation. The results observed are tabulated in Table 1. Various organic matters in soil have been reported to help the degradation of the dye [49]. Presence of resident microorganisms in the soil can aid in the degradation of the dye mixture (set D), but they could not decolourize the dyes by themselves (set C) due to its inability to produce azo-dye reducing enzymes. Another explanation is bacteria need to be adapted to the pollutant

Table 3
Phytotoxicity of mixture of dyes (100 mg l^{-1}) and its degraded products extracted after 24 h incubation for *Vigna radiate* and *Triticum asetivum*

	Distilled water		Untreated mixture of dye		Dye-degraded product (24 h)	
	Vigna radiate	*Triticum asetivum*	*Vigna radiate*	*Triticum asetivum*	*Vigna radiate*	*Triticum asetivum*
Germination rate (%)	100	72	56	21	91	68
Plant height (cm)[a]	22.8 ± 1.1	20.8 ± 0.3	12.7 ± 1.0	6.2 ± 1.2	19.5 ± 1.2	18.2 ± 1.3
Root length (cm)[a]	13.0 ± 0.7	11.0 ± 0.2	7.60 ± 0.4	3.7 ± 0.5	10.20 ± 0.5	10.5 ± 0.5
Shoot length (cm)[a]	9.80 ± 0.5	9.8 ± 0.4	5.10 ± 0.6	2.5 ± 0.6	9.30 ± 0.8	7.70 ± 0.6

[a]Values are mean ± standard deviation.

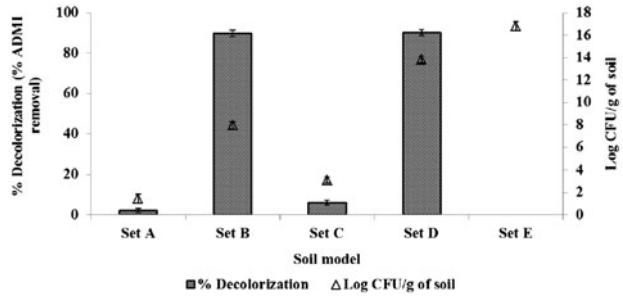

Fig. 6. Decolourization by consortium ASD and cell growth correlation during simulated microcosm studies, (A) sterile soil with dye, (B) sterile soil with dye and consortium ASD, (C) nonsterile soil amended with dye mixture, (D) nonsterile soil with dye and consortium ASD, and (E) sterile soil (without dye) inoculated with consortium ASD.

before they can degrade it [50]. Similar level of decolourization was observed in set B and in set D indicating no significant effect of indigenous soil micro-organism on the test consortium. Consortium ASD grew well in the simulated microcosms while decolourizing the dye which is evident from the increase in the CFU counts (Fig. 6). These results of the microcosm studies reveal the role and efficiency of consortium ASD to decolourize dye.

4. Conclusions

The consortium ASD gave enhanced decolourization of mixture of dyes as compared to pure cultures, signifying a synergistic interaction among various bacterial morphotypes of ASD. Oxidase and reductase enzyme activity during dye decolourization suggest their involvement in the degradation process. Significant reduction in color, COD, and TOC within 24 h incubation indicates the utilization of the dye mixture by the consortium. NMR, FTIR, and GC–MS analysis of extracted end-products confirmed the biodegradation of all the dyes in the mixture. Phytotoxicity verified the formation of nontoxic degraded metabolites. Efficient decolourizing activities even at high salt and dye concentration, it is proposed that consortium ASD has a prospective application in the biotransformation of textile wastewater.

Supplementary material

The supplementary material for this paper is available online at http://dx.doi.10.1080/19443994.2015.1124345.

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