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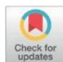

Original Article

Evaluation of Bacterial Consortium and Optimization of Growth Parameters for Effective Decolorization of Azo Dye Reactive Red 120

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Abstract

Introduction: The effluents produced as a result of the dyeing process, especially by textile industries are a major threat to sustainable environmental development. Several challenges are observed in the treatment and disposal of complex azo dyes like Reactive Red 120 (RR120). The aim of the present study was to optimize the dye decolorization/degradation process by bacterial consortium.

Materials and Methods: The consortium consisted of three potential azo dye degraders i.e., *Shewanella haliotis* RDB_1, *Shewanella putrefaciens* RDB_2, and *Aeromonas hydrophila* RDB_3. It was prepared in 1:1:1 ratio and was named as RAR. This consortium was optimized under several nutritional and physicochemical parameters for effective decolorization of RR120.

Results: Complete decolorization of 50 ppm RR120 was achieved with 10% inoculum of 1.0 OD_{540nm} in 3% Yeast Extract (YE) medium under static conditions in 4 h. The optimum decolorization was observed between pH 7-8 and temperature 30°C-35°C. However, the consortium RAR showed significant activity between a pH range of 6-10, temperature 25°C-45°C and NaCl concentration up to 10%. The electron acceptors like nitrate and nitrite salts, and electron donors like urea and casamino acids negatively affected the decolorization rate of RR120. The sugars and organic acids failed to support decolorization in M9 medium. However, a varying effect was observed in the 3% YE medium. Soymeal peptone (prepared in distilled water) supported considerable decolorization but was not as effective as 3% YE medium.

Conclusions: Considering the above features and tolerance of consortium RAR to varied range of pH, temperature and NaCl concentration, it may be a suitable candidate for biodecolorization of textile effluents.

Keywords: Consortium, Decolorization, Optimization, Physicochemical, Sustainable, Textile Effluents

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Introduction

The ever increasing demand for synthetic dyes in major industrial sectors has subsequently led to the generation of tremendous amounts of waste effluents. Over 100 tons of used dyes are discarded into the water bodies from textile, cosmetics, pharmaceuticals, fashion, food and paper industries, globally.[1] The resulting pollution severely compromises the aquatic ecosystem by hampering the light penetration, and thus the process of photosynthesis.[2] The problem is further complicated due to the mixing of these complex dyes with by-products of industrial processes. These by-products, including acids, bases, dispersants and heavy metals, result in high BOD and COD values making the treatment and bioremediation of dye effluents an ardous task.[3] Moreover, several dyes pose additional concern due to their toxicological profile.[4] Azo dyes, contributing to 70% of industrially used dyes, are among such complex dyes, and require specific disposal treatment due to their heat stability and resilience to chemical and biological degradation protocols.[5,6] Azo dyes, preferred industrially due to their cost effectiveness and quality, is thus posing a severe problem for the sustainability and safety of the environment.

To address the issues with the disposal and bioremediation of dye effluents, several physicochemical treatment techniques do exist. These include coagulation/flocculation, precipitation, filtration (using sand), and adsorption. They are simple techniques, however, these strategies pose significant drawbacks due to their high energy and cost requirements. Also, high amounts of chemicals are required for processes like coagulation/flocculation and continuous pH monitoring is essential for precipitation. Moreover, they generate toxic sludge, and are ineffective against recalcitrant coloured effluents.[7] As a result, hopeful and practically sustainable strategies have been encouraged with biodegradation techniques involving the use of micro-organisms. These techniques offer advantages of environmental protection through natural metabolic mechanisms of environmental isolates capable of detoxifying and degrading dyes into harmless products.[8] Another advantage of microbial dye degraders is their ability

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of mineralization of dye and reduced production of sludge.[9] Furthermore, the dye degraders can be utilised for the disposal of an array of dye molecules, making the process relatively inexpensive and more practical. However, they do pose limitations concerning high concentrations of dyes in industrial effluents and their toxicity that challenges the survival of bacteria. Also, several studies, including our previous reports, indicate that the bacterial dye degraders require a suitable co-substrate as a carbon and nitrogen source to induce decolorization and/or degradation of dyes.[9-12] Inspite these limitations, the biological techniques present them as the most effective strategy to challenge the widespread pollution due to dye effluents.

The environment is home to millions of bacterial species and it is logically evident that they work, as well as depend, on the metabolic processes of each other to survive in a suitable niche. Hence, in order to mimic the environmental process, we studied the effect of bacterial consortia, of three previously reported bacterial dye degraders, in the current study. For this purpose, a high molecular weight, ditriazine and dinaphthalene nuclei possessing diazo bond linked, sulphonic sodium salt of Reactive Red 120 (RR120) was selected, due to its complexity. The main objective of our study was to optimise the dye decolorization/degradation process by bacterial consortium.

Materials and Methods

Dye Degrading Bacteria and Designing of Consortia

Three bacterial isolates i.e., *Shewanella haliotis* RDB_1 (LK-1), *Shewanella putrefaciens* RDB_2 (DL-1) and *Aeromonas hydrophila* RDB_3 (LK-2) were isolated and optimised for their dye degrading potential under varying nutritional and physicochemical conditions in previous studies.[10-12] The inoculum for bacterial consortium was prepared by mixing these individual pure cultures in different combinations to deduce the consequence of their concerted metabolism on the efficiency of RR120 decolorization. The pure culture suspensions were mixed in combinations of two isolates in 1:1 ratio to obtain three different consortia, and another of three isolates in 1:1:1 ratio (by volume) to obtain a consortium.

Quantification of RR120 Dye Decolorization by Bacterial Consortia Using UV Visible Spectrophotometer

A 5% v/v (0.2 OD_{540nm}) inoculum of the above bacterial consortium was inoculated in 20 ml Nutrient Broth (NB) containing 50 ppm RR120 and was incubated under static conditions at 30 °C for qualitative assessment of the decolorization (visually) under various experimental conditions. The aliquots (4 ml) from decolorized media were centrifuged at 10,000 rpm/20 min (REMI PR 24 centrifuge) to separate the bacterial biomass. The cell pellet was suspended in 4 ml of methanol to extract any dye adsorbed on the cell surface. The extent of dye degradation was quantified by using a spectrophotometer (Systronic, double beam spectrophotometer 2203) within the UV and visible spectrum (200 nm to 800 nm). The dye decolorization percentage and the rate of decolorization were expressed as equations 1 and 2, respectively.[9-13]

$$\%\ \text{decolorization}\ (\%\ \text{D}) = (\text{A} - \text{B})\ \text{x}\ 100 \qquad \text{Equation 1}$$

Where, A and B represent the absorbance values of the un-inoculated media control and the decolorized supernatant of media inoculated with bacterial consortium respectively.

$$\text{Rate of decolorization}\ (\mu\text{g/L/h}) = \frac{\text{C x \%D}}{\text{V x t}}\ \text{x}\ 1000 \quad \text{Equation 2}$$

Where, C is the initial concentration of dye (mg/L); % D is the dye decolorization (%) after time (t (hour)) and V is the volume of test sample

Optimization of Process Parameters for RR120 Decolorization by Bacterial Consortia

The optimization of process parameters for azo dye RR120 decolorization by bacterial consortia was performed by using the one factor at a time approach. In this approach, one parameter is varied at a time while keeping the pre-optimized parameter as constant.[14]

Optimization of Media and Its Components for Decolorization of RR120 by Bacterial Consortia

In order to optimize the decolorization of RR120 by bacterial consortia, seven different media were screened to determine a suitable growth medium for bacteria consortium. They included Mineral Salt Medium (MSM), NM9 medium, M9 medium, M9 with 1% Yeast Extract (YE), Synthetic Medium (SM) with 0.1% YE, NB and Luria Bertani Medium (LB).[15-18]

Since every component of nutrient medium may act as a vital factor for dye decolorization/degradation, the effect of individual components (of optimized media) were further studied by deletion, substitution and addition assays. In these assays, the media components were omitted, substituted or added, one by one, to study their effect on dye decolorization.

Effect of Biomass of Bacterial Consortium on Decolorization of RR120

The optimum biomass (i.e., O.D and inoculum size) of individual test cultures, optimized in previous studies,[10-12] were mixed in different combinations to obtain a consortium. The different optical densities (0.2, 0.4, 0.6, 0.8, 1.0, and 1.5) and inoculum size (1%, 2%, 4%, 6%, 8% and 10% v/v) of these consortia were then inoculated in the optimised medium to study their effect on the decolorization of RR120.[19,20]

Effect of Yeast Extract and Nacl Concentration On Decolorization of RR120 Bacterial Consortium

The effect of different concentrations (1-6%) of YE prepared in distilled water (i.e., YE medium) was studied on decolorization of RR120 by bacterial consortium.[21] Moreover, considering the presence of high concentration of sodium chloride (NaCl) in textile effluents, where it is commonly used as a mordant, the tolerance of bacterial consortia to NaCl (1-10%) were also observed in optimised medium.[22]

Effect of Physicochemical Parameters on Decolorization of RR120 by Bacterial Consortium

For optimization of physicochemical parameters, the effects of aeration (i.e., static and shaker conditions), pH (4, 6, 7, 8, 9 and 10 unit) and temperature (15 ℃, 25 ℃, 30 ℃, 35 ℃, 45 ℃ and 55 ℃) were studied on decolorization of RR120 by bacterial consortium under previously optimised culture conditions.[23-25]

Effect of Electron Acceptor on Decolorization of RR120 by Bacterial Consortium

The impact of salts like potassium nitrate, potassium nitrite, ammonium nitrate, sodium nitrate and sodium nitrite functioning as alternative electron acceptors was observed in previously optimised mediums.[26]

Influence of Various Chemically Defined and Undefined Cosubstrates as Electron Donors On Decolorization of RR120 by the Bacterial Consortium

The ability of bacterial consortium to decolorize RR120 effectively was screened in the presence of electron donors like 1 g% sugars (glucose, galactose, raffinose, mannose, ribose, fructose, sucrose, mannitol, inulin, trehalose, lactose, maltose, L-arabinose and xylose), 1% organic acids (sodium pyruvate, sodium succinate, sodium lactate, sodium malonate, trisodium citrate dihydrate, sodium acetate, sodium formate and sodium tartarate), 1 g% amino acids (L-lysine monohydrochloride, L-arginine, L-glutamine, L-serine) and urea (1%, 2%, 3%, 4%, 5%).[6,19,27,28]

The effect of chemically undefined/ complex cosubstrates (electron donors), like casamino acids (0.5%, 1%, 1.5%, 2%) and soymeal peptone (1%, 2%, 3%, 4%, 5%, 6%), were also studied as alternatives to YE in distilled water (pH 7.4).[29]

Post-Hoc Statistical Analysis of Data

All the experiments of optimization studies were conducted in triplicates and the results have been represented as mean ± Standard Deviation (SD). The statistical analysis was carried out using the Open Source R Software. The dependent variables with $p<0.05$ were considered to be statistically significant in comparative studies between the pure cultures and bacterial consortium. The Tukey pairwise comparison was used to validate the difference in the mean values. The R-squared values were used to study the relationship between the bacterial consortium and the above optimization parameters.

Results and Discussion

Screening of Consortia for the Decolorization of RR120

Figure 1 represents the four different bacterial consortia assessed for their dye decolorization ability in the current study. It was observed that the consortium of two cultures each, prepared in 1:1 ratio with LK-1 and LK-2, and another with LK-2 and DL-1, exhibited low decolorization rates of 42.77 mg/L/h and 38.55 mg/L/h respectively. In contrast, the consortium of LK-1 and DL-1 yielded a higher rate of RR120 decolorization (64.01 mg/L/h). However, the most effective decolorization rate of 75.89 mg/L/h was achieved with the bacterial consortium containing a combination of all three isolates in 1:1:1 ratio. Hence, the most efficient consortium of three isolates was designated as RAR, and chosen for further optimization studies.

Characteristically, LK-2 was observed to be a slow degrader and hence was presumed to negatively influence the rate of decolorization of RR120 in consortium. However, interestingly, none of the dye degrading bacteria exerted a negative effect on co-inoculation. Both consortia containing LK-2, exhibited higher rates of decolorization as compared to the respective pure cultures i.e. LK-1 (27.72 mg/L/h) and DL-1 (18.79 mg/L/h) as reported in earlier studies.[10-12] The probable effectiveness of consortium RAR to decolorize RR120 may be due to the metabolic efficiency of LK-1 and DL-1, and their synergy and/or co-metabolism with LK-2.[30,31] It has been suggested that the biotransformation of dye by one of the bacteria in a consortium may lead the biodegradation process by allowing other organisms to utilize the intermediate and breakdown products, resulting in increased efficiency of dye degradation.[32,33] Also, the individual strains in consortia may produce specific enzymes leading to ordered coordination of their metabolism to degrade dyes efficiently.[34] Moreover, the degradation of dye molecules may begin at several points instead of one, in a medium containing a consortium, eventually speeding up the overall process of azo dye decolorization.[35]

In a similar study, the consortium of PMB11 comprising of three different bacteria was developed by Patil et al.,[36] which was capable of 99% decolorization of RR120 within 12 h. In another study, two consortia PsGo and StSp were constructed by mixing individual cultures in 1:1 ratio for enhanced decolorization of RB5.[37] Also, a moderately halophilic consortium was created using the genera *Halomonas and Escherichia* for degradation of Acid Orange 7 under saline conditions.[4] The decolorization of various reactive dyes has also been studied by using consortium of five microbial isolates in 1:1 ratio.[38]

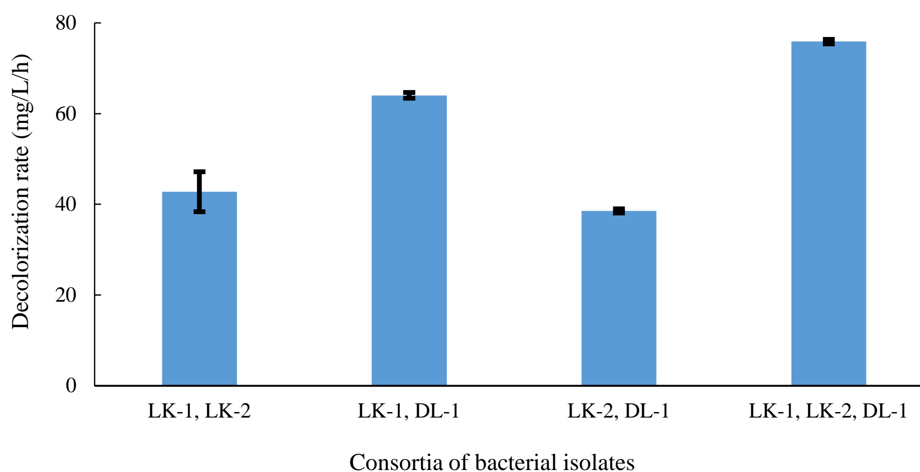

Figure 1. Screening of Various Combinations of Individual Cultures for Decolorization of RR120.

Optimization of Process Parameters for RR120 Decolorization by Consortium RAR

Optimization of Media and Its Components for Decolorization of RR120 by Bacterial Consortia

Our previous studies carried out with pure cultures demonstrated best decolorization activity in 3% YE medium. However, in order to study the effect of other inorganic and organic nutritional sources on the bacterial consortium RAR, we carried out the decolorization assay in various synthetic, semisynthetic and complex nutrient media. Figure 2 represents the effect of these media on dye decolorization by bacterial consortium RAR. The LB medium was found to be most effective with 65.93 mg/L/h removal of RR120. This was much higher than the individual pure cultures of consortia.

Two different media containing YE as one of the nutrients i.e., SM with 0.1% YE and M9 with 1% YE also supported dye decolorization which occurred at the rate of 62.50 mg/L/h and 64.44 mg/L/h respectively. The synthetic media like M9 and MSM, with 50 ppm RR120 as the solo source of carbon and energy, were inefficient in decolorizing the dye as evident with the decolorization rates of 0.37 mg/L/h and 0.02 mg/L/h respectively. The efficacy of YE as a nutrient source was further strengthened based on the deletion assay (Figure 3). It was observed that the deletion of YE from LB medium drastically reduced the rate of decolorization (by 85.63%), by bacterial consortium RAR. Moreover, substituting 0.5% YE with 0.5% meat extract resulted in 59.15% reduction in decolorization rates, whereas substituting other nutrients i.e., 1% tryptone with 1% peptone reduced the decolorization rates by only 5.43%. The deletion of tryptone and NaCl from LB medium also had a minor negative effect on decolorization, with 8.6% and 3.01% reduction respectively. Furthermore, the addition of 0.5% meat extract or 1% peptone to LB medium slightly increased the dye decolorization rates by 0.62% and 0.41% respectively. The significant dependence of the rate of decolorization on YE concentration was also apparent with a higher decolorization rate in M9 medium, containing 10 times higher YE concentration, as compared to the SM medium. The complex medium i.e., NB containing organic nutrient sources, other than YE, also failed to efficiently decolorize RR120.

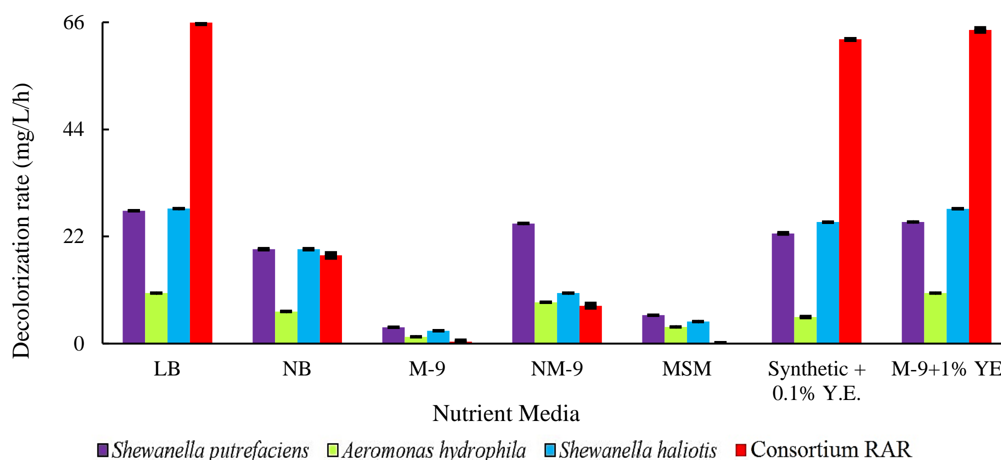

Figure 2. Comparison of Screening of Nutrient Media for RR120 Dye Decolorization by Individual Isolates and Consortium RAR.

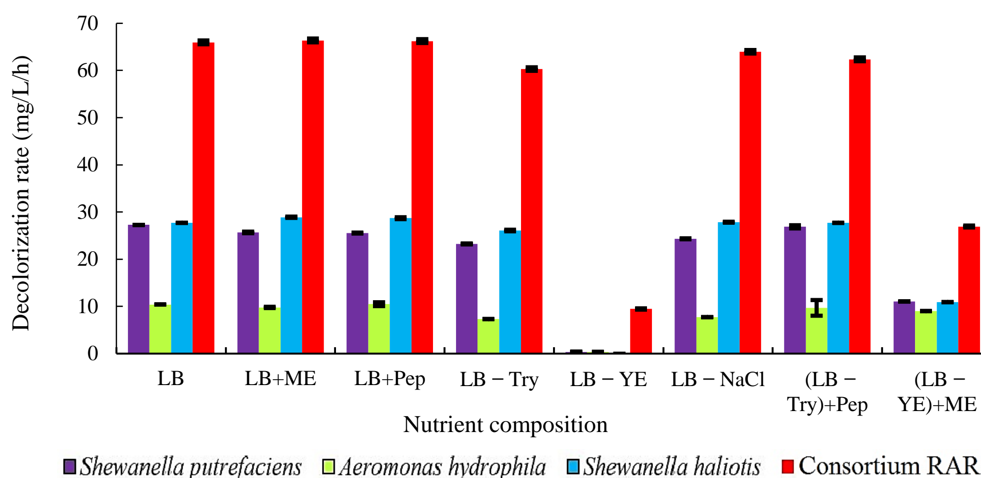

Figure 3. Comparison of The Effect of Substitution, Addition and Deletion of Various Components of the Best Medium on Decolorization of RR120 by Individual Isolates and Consortium RAR.

Thus, based on the observations of optimized nutrient sources, it may be suggested that the bacterial consortium RAR requires a co-substrate like YE for efficient decolorization of RR120 dye. Moreover, the presence of some unidentified nutritional component(s), present in YE and absent in other organic sources, was critical for RR120 dye decolorization by bacterial consortium RAR. Also, the insignificant decolorization of RR120 in M9 and MSM confirmed the inability of bacterial consortia RAR to exploit RR120 as the sole carbon and energy source.

Similar to our study, the efficiency of YE over beef extract, peptone, glucose and tryptone have been reported by previous studies.[39,40] Recently, another study demonstrated the efficiency of 0.4% YE and glucose in BHM for azo dye decolorization of five textile dyes by bacterial consortium.[38] In another study, 0.1% of each glucose and YE in MSM was used by Cao et al. during decolorization of Direct Blue 2B by the consortium YHK.[41]

Effect of Biomass of Bacterial Consortium on Decolorization of RR120

The initial cell mass significantly affects the decolorization process. In the current study, 10% v/v inoculum of 1.0 OD_{540nm} resulted in optimum decolorization of RR120 (65.39 mg/L/h) by consortium RAR (Supplement Figure 1, 2). Similar to the observations of individual cultures, the rate of decolorization improved with a rise in OD_{540nm} of consortium RAR. Also, at optimum biomass, the rate of decolorization by consortium RAR (124.67 mg/L/h) was significantly greater than that of individual isolates (20.60 mg/L/h–90.30 mg/L/h).[10-12] The regression analysis indicated an increase in the decolorization rate by 38.96 mg/L/h up to 1.0 OD (R^2 = 0.8447) and 5.38 mg/L/h up to 10% v/v inoculum size (R^2 = 0.9216) for consortium RAR. The Tukey pairwise comparison test ($p<0.05$) further suggested a dissimilar behaviour of consortium RAR and pure cultures, for the decolorization of RR120 with respect to OD_{540nm} and inoculum size.

The higher cell densities lead to the utilization of electrons, from metabolism of co-substrates, for reduction of oxygen (since it is a preferred electron acceptor in the bacterial electron transport chain) instead of azo bonds of RR120.[42,43] This leads to a lower rate of decolorization, as observed above 1.0 OD_{540nm}. Hence, higher inoculum size (above 10%) was not tested in our study. The efficiency of dye decolorization by consortium RAR may be due to the collective metabolism of individual isolates that positively complemented the decolorization of RR120. It is also possible that the fast decolorizing isolates like LK-1 and DL-1 may be responsible for reducing the azo bonds of RR120 to produce partially degraded intermediates that were utilised by slow degrader i.e. LK-2.

Different strategies have been followed by researchers to optimize the biomass for efficient degradation of dyes. For instance, a 2% consortium comprising of *Moraxella osloensis*, *P. aeruginosa*, *Citrobacter freundii*, *Bacillus* sp., and *Bacillus thuringiensis* was ideal for the decolorization of the dye Acid Blue.[44] In another study, an inoculation cell density of 1.0 ± 0.02 OD_{600nm}, of a bacterial consortium, was used in 50:1 medium to inoculum ratio by Khalid et al.[42] They reported degradation of 100 μM 4-nitroaniline along with other dyes such as AR88, RB5, DR81 and Disperse Orange-3.

Optimization of Yeast Extract Concentration for Decolorization of RR120 by Bacterial Consortium RAR

The consortium RAR showed no preference for RR120 as a sole source of nutrient, as confirmed by observations in M-9 medium (Figure 2) that showed 3.61% decolorization in 24 h. Hence, the supplementation of a co-substrate was essential which can undergo oxidation to generate reducing equivalents to complement the reduction of azo bonds of the dye RR120. Since YE considerably improved the rate of dye decolorization,

varying concentrations were tested to determine its optimum concentration. It was observed that 3% YE was equivalent to LB medium used initially for optimization of nutrient parameters (Supplement Figure 3). It can be concluded that the critical factor acting as an electron donor in 3% YE may be the same as the one present in LB medium. The regression analysis ($R^2 = 0.8677$) indicated an increase in the decolorization rate by 3489.78 mg/L/h up to 3% concentration for every 1% increase in YE concentration for consortium RAR. The Tukey pairwise comparison test ($p<0.05$) suggested that the consortium RAR was dissimilar to LK-2 but similar to LK-1 and DL-1 with respect to the decolorization of RR120 under various concentrations of YE.

With a further increase in the concentration of YE (above 4%), a reduced decolorization rate was observed. Similarly, other studies have also reported the stability of decolorization rate[45] or a negative effect[46,47] on increasing YE concentrations. This may be due to the detrimental effect of higher concentrations of micro or macroelements in YE that makes the medium hypertonic or toxic. In contrast to our study, the efficacy of decolorization remained unchanged with increasing concentration of YE (0.1–5 g/L) in Bushnell Hass medium along with glucose while studying decolorization of Reactive Violet 5R by acclimatized natural consortium SB4.[45]

Although it is unclear whether YE acts as an electron shuttle/ redox mediator or supports bacterial growth, it can be argued that it serves both purpose. This is because, it has been suggested that the vitamin B_2 component of YE is crucial for azoreductase activity of the bacteria, and other components, including vitamin B_2, and also stimulates bacterial growth.[48] Other than YE, redox mediators like ethyl viologen, anthraquinone-2, 6- disulfonate, methyl viologen etc., have been proposed to augment azo dye degradation.[49,50] However, the process requires an additional co-substrate making the biotreatment economically unfeasible. In contrast, YE can act as cosubstrate as well as redox mediator for metabolism of dyes and thus serve as an ideal candidate for this role.[51]

Optimization of NaCl Concentration for Decolorization of RR120 by Bacterial Consortia

The dye was degraded optimally (124.23 mg/L/h) by the consortium RAR, as well as its individual members, when the culture medium was supplemented with 1% NaCl concentration (Figure 4). Beyond this concentration, the individual isolates showed gradual reduction in dye decolorization potential. However, the consortium RAR could tolerate up to 10% NaCl concentration. Moreover, it could decolorize 50ppm dye in culture medium containing 5% NaCl in 5 h (Figure 5). With further incubation for 24 h, the consortium RAR could decolorize RR120 even in culture medium containing 10% NaCl (Figure 6). Among pure cultures, LK-1 and DL-1 showed significant decolorization of RR120 between 1%–5% NaCl concentrations, whereas LK-2 was tolerant to only up to 1% NaCl concentration. Hence, in comparison to pure cultures the consortium was much superior in terms of its salt tolerance. The regression analysis ($R^2 = 0.8646$), indicated a decrease in the decolorization rate by 1287.12 mg/L/h for every 1% increase in NaCl up to 10% NaCl concentration, for consortium RAR. The Tukey pairwise test ($p < 0.05$) indicated similarity in the activity of the consortium RAR and pure cultures for the decolorization of RR120 under various concentrations of NaCl.

In textile industries, dyeing efficiency has improved by adding inorganic salts as fixing agents. The released dye bath waste waters contain elevated concentrations of salts such as NaCl. Although NaCl is essential for the cellular activity, higher concentrations increase the osmolarity of the effluent that affects the cellular metabolism, by insufficient NADH production, and eventually compromises the degrading ability of the bacteria.[6,52]

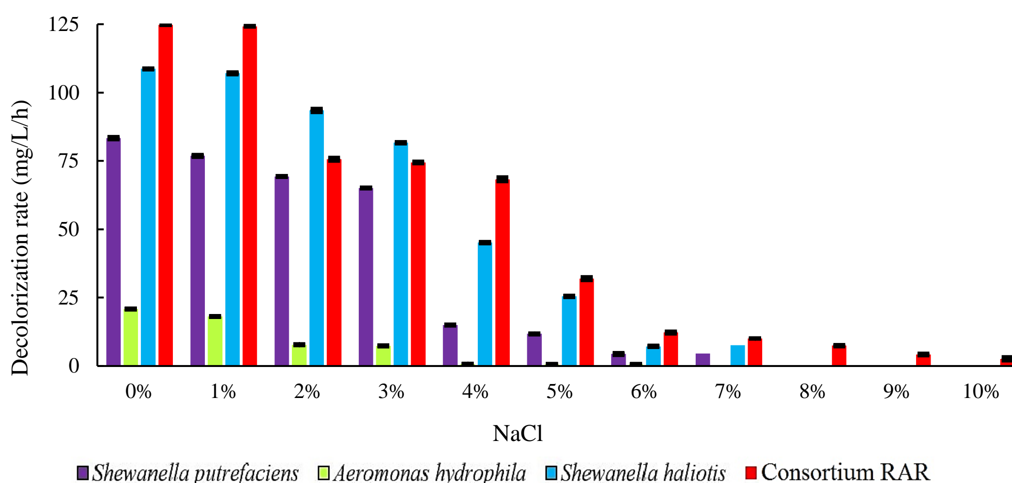

Figure 4. Comparison of the Effect of Various Concentration of NaCl on RR120 Decolorization by Individual Isolates and Consortium RAR.

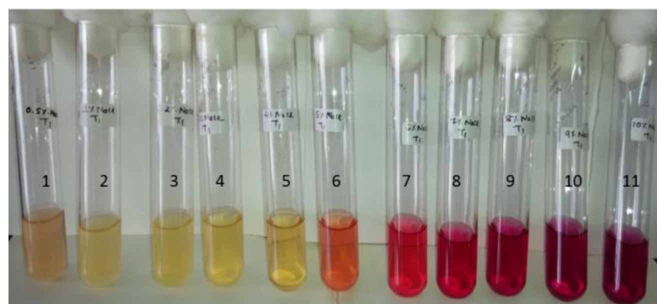

Figure 5. Decolorization of RR120 in 3% YE Medium by Consortium RAR under Various Concentrations of NaCl after 5h. The above figure shows the extent of decolorization of RR120 in Tube 1 containing 0.5% NaCl and Tubes 2-11 containing 1-10% NaCl respectively.

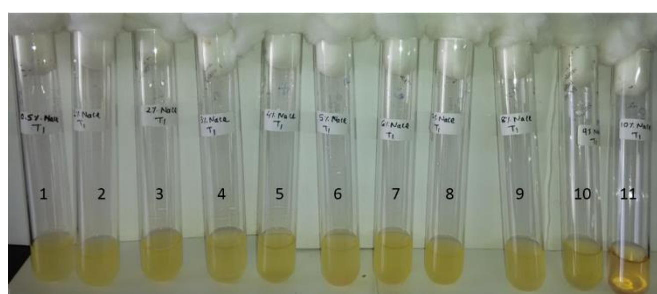

Figure 6. Decolorization of RR120 in 3% YE Medium by Consortium RAR under Various Concentrations of NaCl After 24 h. The above figure. shows the extent of decolorization of RR120 in Tube 1 containing 0.5% NaCl and Tubes 2-11 containing 1%–10% NaCl respectively.

In textile industries, dyeing efficiency has improved by adding inorganic salts as fixing agents. The released dye bath waste waters contain elevated concentrations of salts such as NaCl. Although NaCl is essential for the cellular activity, higher concentrations increase the osmolarity of the effluent that affects the cellular metabolism, by insufficient NADH production, and eventually compromises the degrading ability of the bacteria.[6,52] Saline environment also inhibits catalysis by degradative enzymes, and alters the metabolic pathways of a bacterial consortium.[53] It is reported that azo dye reduction is inhibited above 3000 mg/L concentration of NaCl.[52] In order to effectively degrade complex dyes, it is necessary to select a salt tolerant microbial strain. These strains work either by the production of stable enzymes that help the degradation or osmoprotectant molecules that help in their survival.[6]

Few salt tolerant microbial consortia have been reported in literature. Similar to our study, Guo et al.,[6] indicated a decline in the decolorization of 100 mg/L azo dye Metanil yellow, by consortium M1, with a rise in NaCl concentration from 1%-10% in 6 h. Chen et al.,[54] also confirmed a contrary relationship between decolorization rate and Z. The halophilic consortium VN.1 decolorized the dye RB220 with an insignificant decrease in the rate of decolorization at salt concentration above 7% in a study carried out by Patel and Bhatt.[55]

Effect of Physicochemical Parameters on Decolorization of RR120 by Bacterial Consortium

The optimum decolorization ability of consortium RAR was observed under static conditions (Supplement Figure 4) in 3% YE medium adjusted to pH between 7-8 (Supplement Figure 5) and a temperature range of 30-35 °C (Supplement Figure 6). However, significant decolorization ability was observed over a broad pH range of 4-10 and temperature range of 25-45 °C. Under aerobic conditions, the individual cultures showed comparatively less reduction in decolorization rates (3.99%-18.97%).[10-12] However, for the consortium RAR, the rate of decolorization for RR120 decreased by 50.65% under aerobic conditions. The individual members of the consortium RAR also demonstrated significant decolorization of RR120 over a narrow range of pH 6.0-9.0 for LK-1, pH 6.0-8.0 for LK-2 and pH 7.0-9.0 for DL-1 within 4 h. On an extended incubation for 24 h, 100% decolorization was not observed by individual isolates between pH 5.0-9.0.[10-12] The consortium, on the other hand, showed 100% decolorization of RR120 dye in 3% YE medium between pH 6.0-8.0 within 2.5 h (Supplement Figure 7), and between pH 5.0-9.0 in 24 h (Supplement Figure 8). The regression analysis indicated an increase in the decolorization rate by 80.98 mg/L/h (R^2 = 0.8452) for every 1.0 unit increase in the pH of the medium up to pH 8.0, and 19.01 mg/L/h (R^2 = 0.9309) for every 1°C rise in temperature up to 35 °C, by consortium RAR. The Tukey pairwise comparison (p<0.05) test indicated that the consortium RAR was dissimilar to LK-2, and similar in activity to LK-1 and DL-1, for the decolorization of RR120 under various pH; whereas it showed similar activity to all three pure cultures for decolorization of RR120 at various temperatures.

Dye decolorization being a metabolic process of bacteria, is highly influenced by conditions such as pH, temperature and availability of oxygen. This is also why several studies have reported neutral pH[56-59] and temperature range of 30-35 °C[34,44,60] as optimum for dye degradation by bacterial isolates and consortia. Also, to the best of our knowledge, there is no evidence of acidic pH being optimum for bacterial dye decolorization. The pH of the medium affects the surface charge of bacterial cells that is responsible for the uptake of dye through the cytoplasmic membrane.[61] The pH as well as temperature fluctuations may alter the structure of dye degrading enzymes resulting in denaturation and inactivation of the protein.[54,62] Since consortium RAR showed significant decolorization ability over a wide pH and temperature range, it may be a suitable candidate for bioremediation of textile effluents. This is because the dye bath effluents have a temperature of 40 °C and are alkaline in nature. The reduced

rate of decolorization at elevated temperatures (55 °C) can be linked to cellular viability and denaturation of azoreductases.[63] Also, since aerobic conditions lead to decreased decolorization by diverting electrons for reducing O_2 instead of azo bonds by azoreductases, the static requirement by consortium RAR for decolorization will be helpful in reducing the overall energy requirement necessary for operating aerobic treatment plants.

Similar to our study, the halotolerant consortium VN.1 composed of six strains showed 7.1 times enhanced decolorization of Reactive Blue 220 at 34 °C and pH 8.0 in the absence of O_2 as compared to shaker conditions. Moreover, it was tolerant to a wide-range of pH of 6.0–10.0 and temperatures of 26-40 °C.[55] In a different study, the dye Rubine GFL reduced efficiently (with 94% decolorization) when the fungal consortium AP was grown at 37 °C within a pH range of 6.5-8.5 in 37 h.[64] A bacterial consortium with strains *Zobellella taiwanensis* strain AT 1-3 and *Bacillus pumilus* strain HKG212 decolorized the dye Reactive Green 19 with maximum potential (94.10%) at pH 8.0 and 30 °C in 24 h. It also showed significant decolorization above 55% between pH of 5.0-10.0.[65] A recent study reported 95.43% decolorization of 100 mg/L Metanil Yellow dye at pH 7.0, 30 °C under static conditions in 6 h by consortium M1 comprising of *Pseudomonas* sp, *Lysinibacillus* sp, *Lactococcus* sp, and *Dysgonomonas* sp.[6]

Effect of Alternative Electron Acceptors on Decolorization of RR120 by RAR

It was observed that nitrate ions, in concentrations of 0.1%, 0.5% and 1.0%, negatively affected the decolorizing ability of consortium RAR as compared to the same concentration of nitrite ions (Supplement Figure 9-13). The rate of decolorization of RR120 was very low in the range of 0.02 mg/L/h to 5.45 mg/L/h in the presence of these ions as compared to 124.45 mg/L/h in 3% YE in the absence of these ions. It was also evident in our study that the coupling ions in nitrate salts may influence RR120 decolorization since the pattern of inhibition was different for the three nitrate salts. This investigation clearly indicated that nitrate and nitrites were preferred by consortium RAR as alternative electron acceptors over azo bonds of RR120 for the respiratory metabolism in the absence of oxygen. This may be as a result of both the ions serving as electron acceptors in the periplasm, and its upstream location as compared to RR120 in the electron transport chain of the consortium RAR.[66-68] Also, according to Field et al.,[69] a large amount of energy is made available when electrons are donated from NADH to nitrate. This further increases the preference of nitrate over the azo dye as an electron acceptor.

It was imperative to check the influence of these ions on the decolorization potential of the consortium, since these ions are present in the dye baths and consequently in textile waste water as they are essential for the dyeing process. The results similar to our study were noted by Patil et al.,[36] during the decolorization of Red HE3B by the consortium PMB11. They used two nitrate salts i.e. ammonium nitrate and potassium nitrate in synthetic medium with YE to supply extra nutrients for dye degradation. Their results clearly showed a reduction in dye decolorization from initial 97% to 67% and 71% in the presence of ammonium nitrate and potassium nitrate respectively.

Effect of Various Chemically Defined Cosubstrates/Electron Donors on RR120 Decolorization by RAR

In the present study, a variety of chemically defined as well as complex electron donors which can act as cosubstrates for RR120 decolorization were studied. Comparatively insignificant decolorization was observed in M9-medium containing 1% sugars. The highest rate of decolorization, of only 12.60 mg/L/h, was obtained with maltose. Similar rates of decolorization (12.04-12.54 mg/L/h) was also observed with galactose, mannose, mannitol, sucrose and inulin followed by fructose, ribose, trehalose, and glucose (10.39-11.97 mg/L/h decolorization), whereas, negligible decolorization occurred in the presence of other sugars (Supplement Figure 14). The same sugars efficiently supported the decolorization of RR120 in 3% YE base medium (Supplement Figure 15). The decolorization rate of 142.50 mg/L/h was obtained in the control medium i.e. 3% YE, and similar activity i.e. neutral effect (141.97 mg/L/h-142.56 mg/L/h) was observed for all tested sugars except for raffinose, mannose and ribose. These sugars showed a decolorization rate in the range of 117.47 mg/L/h-134.56 mg/L/h. Similar observations were noted for 1% organic acids and amino acids that showed insignificant decolorization in M-9 medium (Supplement Figure 16, 17). Even in 3% YE medium, considerably less decolorization was observed for these organic acids, except for L-glutamine (141.45 mg/L/h). A 1% urea also failed as a co substrate to decolorize RR120 (Supplement Figure 18). It can be concluded that none of the electron donors used in our study were a favorable cosubstrate for RR120 decolorization as compared to 3% YE.

There is a deficit of carbon atoms in azo dyes due to which it is not easy to biodegrade them without the presence of additional nutrient(s).[70] Azo dye decolorization/ degradation by bacterial consortia can proceed only if certain organic carbon and nitrogen compounds, as cosubstrates, are available in the growing habitats of bacteria. The quality and quantity of these compounds are the key factors for deciding the efficiency of biodecolorization. This holds true as the initial phase in azo dye decolorization is the reduction of azo bond(s) by the enzyme azoreductase. This step requires reducing equivalents in the form of NADH/NADPH generated during energy yielding metabolism carried out by the bacteria using organic compounds.[71] The inability of

urea to support dye decolorization may be explained based on its chemical structure that shows the presence of organic carbon in much less concentration as compared to organic nitrogen. This affects the process of oxidation to supply electrons for the reduction of azo linkages of reactive azo dye. Additionally, the reduced nitrogen in the form of NH_4^+ in cleaved urea may be assimilated by the cells rather than oxidized to support the decolorization process.

The ability to metabolize specific cosubstrates by the bacterial consortia is also decided by the genotype of the bacterium. Some cosubstrates are often more preferred over the others due to their richness with respect to electron concentration or the presence of efficient metabolic enzyme machinery in the dye decolorizing/degrading bacteria. Organic nitrogen nutrients have supplementary augmenting influence on azo dye reduction than carbon nutrients.[70] The enhancement of catalytic activity of azoreductase and sequentially improvement in the decolorization rate with the presence of YE has been studied by Imran et al.[48] Also, the nitrogen sources such as YE, meat extract, peptone and urea, can provide reducing equivalents which can speed up the reduction of azo bonds by bacteria.[70]

Effect of Various Complex Cosubstrates/Electron Donors on RR120 Decolorization by RAR

The results obtained with complex cosubstrate like casamino acids were not promising in comparison with YE, and showed an almost constant rate of decolorization (9.23 mg/L/h-9.93 mg/L/h) of RR120 by consortium RAR (Supplement Figure 19). The rate of decolorization by consortium RAR was also similar to that of individual cultures. Another complex cosubstrate i.e. soymeal peptone showed better activity as compared to casamino acids. The decolorization rate increased from 14.60 mg/L/h at 1% to 55.56 mg/L/h at 5% soymeal peptone concentration (Supplement Figure 20).

The inability of casamino acids to support significant decolorization/degradation of RR120 may be due to the nutritionally deficient chemical composition of casamino acids preparation, which had probably served as a poor electron reservoir due to the insufficient concentration of carbon energy sources. The preparation is mainly a mixture of free amino acids and some very small peptides obtained from acid hydrolysed casein. Usually tryptophan is absent in this preparation. The ready amino acids from casamino acids may be utilized for biosynthetic metabolism of cells and hence not made available as cosubstrates to donate electrons for dye decolorization. Additionally, this preparation does not contain many vitamins as they are destroyed during acid treatment. The concentration of oxidizable carbon being limited in any concentration of casamino acids could also be the reason for giving approximately a constant rate of decolorization over a range of concentrations. On the other hand, though the decolorization potential of RAR increased with increasing the concentration of soymeal peptone, it was not comparable to the activity observed with 3% YE. Since, soymeal peptone is an enzymatically digested product of soybean meal, it may be nutritionally rich, in terms of amino acid composition, as compared to casamino acids. Hence, it may support higher decolorization rates of RR120 by consortium RAR. However, at the same time, the nutritional content of soymeal peptone may not be equivalent to YE. In addition, lower decolorization rates were observed in soymeal peptone as compared to 3% YE medium.

As observed in our study, Patil et al.,[36] also reported the inability of consortium PMB11 to decolorize Red HE3B in synthetic medium without a cosubstrate. On supplementing the synthetic media with different organic carbon and nitrogen supplements like lactose, meat extract, glucose and peptone, the consortium could decolorize the same dye up to 33%, 67%, 70%, and 71% respectively. They also reported YE as the best cosubstrate that supported 97% decolorization of Red HE3B in 24 h.

According to Carliell et al.,[72] reducing equivalents such as NADH, $FADH_2$ are generated during the metabolism of glucose which serve as redox mediators needed for enhanced decolorization of azo dyes. Similarly, Chen et al.,[73] confirmed YE as a superior nitrogen supplement as compared to beef extract, monosodium glutamate, tryptone, peptone, meat extract and urea during the decolorization study of RR198. However, the inhibitory effect of carbohydrates like glucose, during anoxic dye decolorization/degradation had been recognized either due to lower pH by acidic end products of metabolism or due to catabolite repression.[73]

Comparison of RR120 Degradation Ability of Pure Cultures and Consortium RAR and Novelty of the Study

Table 1 summarizes the optimized parameters for RR120 decolorization by the pure cultures and consortium RAR, and the comparison of their respective decolorization rates. The three isolates i.e., *Shewanella haliotis* RDB_1 (LK-1), *Shewanella putrefaciens* RDB_2 (DL-1) and *Aeromonas hydrophila* RDB_3, showed good compatibility with respect to their dye degrading ability, when present as a consortium. To the best of our knowledge, this is the first study reporting *S. haliotis* in a consortium for efficient dye degradation. Moreover, the consortium RAR could decolorize 50 ppm RR120 dye completely within 2.5 h under optimised conditions. Very few studies have reported complete dye degradation of high molecular weight complex dyes like RR120, by bacterial consortium, within such a short time period. In addition, the consortium RAR carried out efficient decolorization over a wide range of pH (5.0-9.0) and temperature (25-45 °C) without any modification in other nutritional or physicochemical parameters.

Table 1. Comparison of RR120 Degradation Ability of Pure Cultures and RAR Under Various Optimized Conditions

	LK-1 (*S. haliotis*)	LK-2 (*A. hydrophila*)	DL-1 (*S. putrefaciens*)	Consortium RAR
Medium	LB	LB	LB	LB
Degradation rate (mg/L/h)	27.71	10.39	27.28	65.93
Static/shaker	Static	Static	Static	Static
Degradation rate (mg/L/h)	27.42	9.51	26.93	76.67
Inoculum size	10% of 1.0 OD_{540nm}	10% of 1.5 OD_{540nm}	10% of 1.0 OD_{540nm}	10% of 1.0 OD_{540nm}
Degradation rate (mg/L/h)	90.30	20.60	69.82	124.67
YE concentration	3% YE	3% and 4% YE	2% and 3% YE	3% YE
Degradation rate (mg/L/h)	101.24	20.83	83.33	124.45
pH	pH 8.0	pH 7.0	pH 8.0	pH 7.0
Degradation rate (mg/L/h)	107.14	20.47	81.27	124.30
Temperature	35 °C	25 °C/30 °C/35 °C	35 °C	35 °C
Degradation rate (mg/L/h)	108.70	20.83	82.98	124.74

Conclusion

The bacterial consortium RAR of three isolates used in the current study exhibited excellent decolorization potential for azo dye RR120 as compared to other published studies. However, the decolorization activity was reduced in the presence of nitrate and nitrite salts. Similarly, the other electron donors were inefficient as compared to 3% YE for RR120 decolorization. It can be hypothesized that the chemically pure compounds like carbohydrates, amino acids and organic acids probably provided purely oxidizable carbon as a limited source of electrons. Similarly, the chemically pure organic nitrogen compound i.e., urea also provided a nitrogen source which can be made useful only if the bacteria possessed urea hydrolysing activity. Hence, the effluents containing high concentrations of the above compounds may interfere with dye decolorization. Inspite of these observations, the observed tolerance to a wide range of pH, temperature and NaCl concentrations may allow its suitability for biodecolorization of dye effluents that are discarded as industrial wastes.

Authors' Contributions

Both the authors made equal contribution to the study design, analysis and literature review. RB wrote the first draft of the manuscript and carried out statistical analysis. RB and KA made appropriate changes to finalize the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Supplementary Materials

Supplementary file 1 contains Figures 1-20.

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