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Microaerophilic decolorization of a textile dye C.I. Reactive Red 31 by indigenous bacterial consortium: Process optimization and enzymatic mechanism

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ABSTRACT

Dyestuff compounds released by textile and dye manufacturing units pose a great threat to the quality of soil-water ecosystems. The present study investigates the ability of bacterial consortium isolated from dye contaminated sites to decolorize a sulfonated azo dye Reactive Red 31. Dye decolorization results, indicated that consortium RV2 under microaerophilic condition decolorized (87.05% of 100 mg/L) dye within 12 h in MSM medium at 37°C. Under oxygen-rich conditions (at 120 rpm), consortium RV2 was incapable for metabolizing dye and only 39.74% (of 100 mg/L) dye was decolorized in the similar time period. The optimum pH and temperature for the dye decolorization were 8 and 37°C, respectively using consortium RV2. A significant increase in azoreductase (14 folds) and tyrosinase (10 folds) activities in the culture broth were obtained after complete decolorization as compared to the initial stage. Initial screening by Plackett–Burman design was performed to select major variables out of eleven media and culture conditions, among which peptone, meat extract, pH and dye concentration were found to have significant effects on decolorization. pH with Prob > F-value of 0.0032 was found to be the most influencing factor followed by concentrations of Peptone (0.0066), Meat Extract (0.0098) and Dye concentration (0.0127) in the medium. The comparison of the response surfaces obtained suggests that peptone and pH along with meat extract plays an important role in dye decolorization, azoreductase and tyrosinase activity. The consortium RV2 possesses the ability to decolorize (99.88%) dye within 12 h under optimum conditions.

Keywords: Reactive Red 31; Bacterial consortium; Azoreductase; Plackett-Burman design; 16S rRNA

1. Introduction

Colored industrial effluents produced by different activities, especially from the textile industries, are a great environmental concern [1,2]. Over 100,000 dyes produced annually, more than 7×10^5 metric tonnes of dyestuffs are manufactured worldwide [3,4]. As some dyes are visible even at lower concentrations of 0.005 mg/L, the presence of dyes is the most evident indicator of water pollution [5]. According to an estimate, approximately 10–15% of the dyes used in dyeing process does not bind with the textile fibres and are released into the water bodies [6]. 2.8×10^5 tons of synthetic dyes are released in textile industrial wastewater worldwide each year [7]. Textile azo dyes are assumed to be electron deficient xenobiotic compounds as they own electron withdrawing groups, generating electron deficiency in the dye molecule, rendering them more resistant to biological degradation [8].

Generally, dyes applied in textile industries (60–70%) are azoic in nature bearing the aromatic ring structures with functional group R-N=N-R′, in which R and R′ are usually substituted aromatic compounds [9]. The environmental and health-related hazard of these aromatic recalcitrant compounds has been recognized, as they possess mutagenic, carcinogenic and toxic properties [10,11]. These issues have raised the urgent need of techniques for their removal from the soil water ecosystem. Many physical and chemical techniques have been effectively projected like ozonation, adsorption, electrochemistry, membrane filtration, ion-ex-

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change and flocculation. However, an operational and economic drawback have favoured the application of biological processes to degrade dye contaminated effluents, since they are cost-effective, eco-friendly and does not generate hazardous sludge [12,13]. Biological treatment technologies involve bacterial treatment, fungal treatment, enzymatic degradation and phytoremediation. In which remediation using bacterial cultures has attained increased momentum as it is faster and effective as compared to fungal based systems and phytoremediation and also more affordable than enzymatic systems [14,15].

The application of consortium in bioremediation has a clear advantage in dye bioremediation due to the availability of richer metabolic network for the bioremediation of contaminated environments [16]. Although reported bacterial consortiums seem to be efficient in dye decolorization, more efforts still should be put on enhancing the decolorizing performance of various efficient microbial consortiums in the low-nutrient environment for the cost-effective process. Among few oxido-reductive enzymes displaying degradative activity of azo dyes, azoreductase enzymes are particularly effective since they function by reducing the azo bonds which is the chromophoric group of colored compounds [17].

Culture condition optimization has been conventionally carried out one factor at a time, determining the effect of one variable while the others are maintained at a certain level. The drawbacks include time consumption and high cost, in addition to ignoring total interactions between medium components. These reasons encouraged researchers to switch for statistical experimental approaches (such as the Plackett–Burman and response surface methodology (RSM) designs), which provide huge information based on only a few experiments [18].

The present study is focused on the bio-decolorization of Reactive Red 31 using isolated and identified bacterial consortium. Physico-chemical conditions and media components have been optimized for enhancing dye decolorization. The role of azoreductase and tyrosinase enzyme was also assessed. Statistical experimental designs (Plackett–Burman design followed by Box-Behnken design) were applied for the optimization of medium components to decolorize Reactive Red 31 dye using bacterial consortium RV2. After neglecting the insignificant variables with the help of initial design for screening (i.e. Plackett-Burman Design), the potent bacterial consortium RV2 was used to decolorize Reactive Red 31 dye by using the Box-Behnken experimental design for the investigation and validation of process parameters and media composition affecting the elimination of sulfonated azo dye.

2. Material and methods

2.1. Materials

Reactive Red 31 (RR31), Reactive Red ME4BL (RRME4BL), Reactive Red RB (RRRB), Reactive Black 5 (RB5), Reactive Red HE8B (RRHE8B), Reactive Violet C2R (RVC2R) and Reactive Blue 220 (RB220) dye used in this study was collected from a dye manufacturing industry in Vatva G.I.D.C., Ahmedabad, Gujarat. All other chemicals utilized in this study were of highest purity available.

2.2. Sampling, screening of bacterial consortium and culture media

Dye contaminated soil and wastewater samples were collected from GIDC area of Vatva, Ahmedabad, India, to acquire acclimatized bacterial strain adapted to catabolize azo dye compounds. Five grams of collected samples were added in Minimal Salt Medium (MSM) amended with 50 mg/L of RR31 and 1.0 g/L Peptone (Fig. 1) under static (microaerophilic) and shaking (120 rpm) conditions and analysed for dye decolorization. Culture flasks showing dye decolorization was further acclimatized by gradually exposing them to increasing dye concentration in fresh culture medium upto 400 mg/L of dye concentration. Bacterial strains were isolated from flasks showing decolorization on Nutrient Agar plate. Three different bacterial consortiums were developed from potent dye decolorizing bacterial strains (data not shown). A potent consortium consisting of five bacterial cultures was selected based on its ability to decolorize RR31 and other tested dyes more efficiently after 24 h of incubation at 37°C under static conditions.

For elucidating cultural components of potent consortium RV2, the isolated bacterial strains were subjected to identification using 16S rRNA technique. The isolated genome of isolated bacterial cultures was subjected to PCR amplification of 16S rRNA using universal primers. A PCR mixture of 20 ng of extracted genomic DNA, 1 µL (100 ng each) of the primers, and 50 µL reaction buffer containing 1.0 µL dNTP mix (2.5 mM each), 1 X Taq buffer A (10 X) and 3 units Taq polymerase was prepared. PCR was performed using a thermocycler. PCR products were examined by 1.0% agarose gel electrophoresis, stained with ethidium bromide and observed under UV transilluminator. The amplified product was purified by gel and obtained products were sequenced.

2.3. Determination of dye decolorization

At regular time intervals, sampling from cultures was carried out and centrifuged at 7000 rpm for 10 min for the separation of bacterial cells. The absorbance of the supernatant was measured spectrophotometrically at the λ_{max} of Reactive Red 31 dye (536 nm). The percentage of decolorization was calculated as follows:

$$\text{Decolorization (\%)} = ((A_{initial} - A)/A_{initial}) \times 100 \quad (1)$$

where $A_{initial}$ was absorbance value of the culture at 0 h, A was absorbance value of the culture at the predetermined time.

2.4. Optimization of nutritional requirement and physico-chemical parameter

In a real sense, biological decolorization of azo dyes should always be conducted through co-metabolism. The bacterial consortium must acquire energy from the oxidation of primary energy source (e.g., organic substrate) for dye biodegradation [19]. Ten mL of consortium RV2 (overnight grown at 37°C), was harvested at 6000 rpm and resuspended in 1.0 mL sterile distilled water (1.0 O.D at 600 nm).

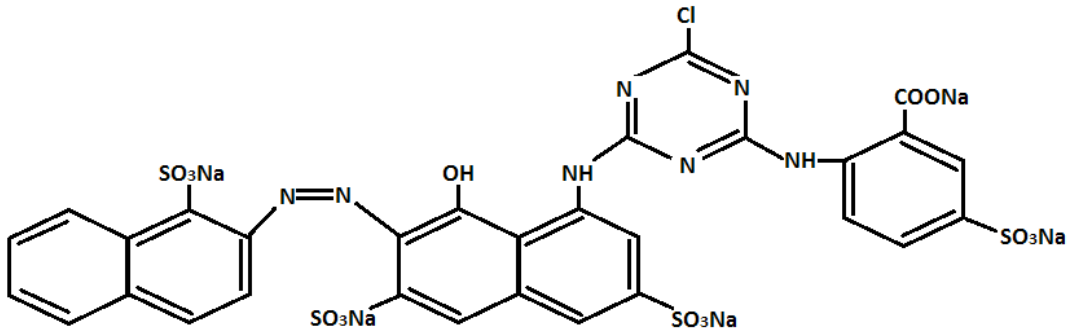

Fig. 1. Chemical structure of Reactive Red 31 (CAS Number: 12237-00-2 and λ_{max}: 536 nm).

The resuspended cell mass devoid of media components were served as inoculum for further studies.

Dye decolorization potential of consortium RV2 was improved by optimizing presence of various co-substrates in the form of various organic (peptone, yeast extract, glucose, starch, malt extract, glycerol, meat extract) and inorganic (ammonium sulfate, urea, EDTA, ammonium acetate, ammonium chloride, ammonium ferrous sulfate) carbon and nitrogen sources.

RR31 decolorization by the bacterial consortium was studied at different physico-chemical conditions. The influence of various parameters such as initial pH (4.0–10.0), temperature (20–55°C), dye concentration (100–1000 mg/L) was determined on RR31 decolorization at 37°C in static conditions.

2.5. Enzymatic studies

2.5.1. Preparation of cell-free extract

The bacterial consortium was activated in Erlenmeyer flasks (250 mL) containing 100 mL sterile medium (RR31 dye added in medium) of pH 8.0, incubated at 37°C for 12 h under static condition and cells were harvested by centrifugation at 6000 rpm for 20 min. The supernatant attained after centrifugation of culture was directly utilized as extracellular enzymes source.

2.5.2. Enzyme assay

The preliminary stage in bacterial decolorization of azo dyes involves reductions in either aerobic or anaerobic condition. This reduction may involve various enzymatic mechanisms, which may be either intracellular or extracellular. Azoreductases, seems to have shown great potential in case of dye decolorization and degradation.

The azoreductase activity was assayed by monitoring NADH disappearance at 440 nm based on the modified procedure described by Kalyani et al. [20]. Reaction mixtures for the standard assay contained in a 2.0 mL (total volume): 50 mM phosphate buffer pH 7.4, 1 mM NADH, 0.25 mM dye solution and 200 µl of enzyme solution. The reaction was started by the addition of NADH followed by monitoring the decrease in color intensity at 536 nm. Tyrosinase activity was determined by the method of Zhang and Flurkey [21]. All enzyme assays were carried out at 37°C, where the reference blanks contained all components except an enzyme. All enzyme assays were run in triplicate and average activity was calculated.

2.6. Experimental design and statistical analysis

The Plackett–Burman experimental design was employed to determine the relative importance of various media and culture components and select the most effective parameters for RR31 decolorization by potent consortium RV2 out of 11 medium and culture components including KH_2PO_4 (A), $(NH_4)_2SO_4$ (B), peptone (C), meat extract (D), $MgSO_4$ (E), NaCl (F), $CaCl_2$ (G), pH (H), temperature (J), inoculum size (K) and dye concentration (L) at two levels: high (+1) and low (−1) (Table 1). This design assumes that there are no interactions between the different media constituents, xi, in the range of variables under consideration. A linear approach is considered to be sufficient for screening.

$$Y = \beta o + \Sigma \beta i xi \ (i = 1, \ldots, k) \quad (2)$$

where Y is the estimated target function and βi are the regression coefficients.

The eleven variables were assessed by twelve experiments and the levels of each variable were determined based on prior experience with the experimental system. Identification of significant variables was carried out by the investigation of the Placket and Burman experiments and their levels were further optimized for enhanced dye decolorization by employing a Box–Behnken design [22]. Experimental design and statistical analysis of data were done by using Design Expert 9 software.

Student's t-test was applied for the identification of the significance level (p-value) of each variable. All the experiments were conducted in triplicates and results mentioned are their mean average. The obtained results were analysed through one-way analysis of variance (ANOVA) Tukey Kramer comparison test.

3. Results and discussion

In recent times, bioremediation using indigenous microbes has become an appealing approach for the eradication of colored pollutant compounds (i.e. dyes) and toxicity from industrial wastewater as compared to other

Table 1
Media components, their contribution and test levels for Plackett–Burman design

Code	Parameter	Levels		Std. Effect	% Contribution
		High level	Low level		
	Constant				
A	KH_2PO_4 (g L^{-1})	0.75	0.25	–26.74	09.30
B	$(NH_4)_2SO_4$ (g L^{-1})	1.0	0.25	–3.29	0.16
C	Peptone (g L^{-1})	1.5	0.0	36.64	19.34
D	Meat Extract (g L^{-1})	1.25	0.0	30.11	13.06
E	$MgSO_4$ (g L^{-1})	0.75	0.25	–2.96	0.13
F	NaCl (g L^{-1})	10	2.5	4.25	0.26
G	$CaCl_2$ (g L^{-1})	0.03	0.01	–3.04	0.13
H	pH	9	5	52.96	40.39
J	Temperature (°C)	37	26	–1.10	0.017
K	Inoculum size (mL)	1	5	4.19	0.25
L	Dye concentration (mg L^{-1})	500	100	–26.40	10.04

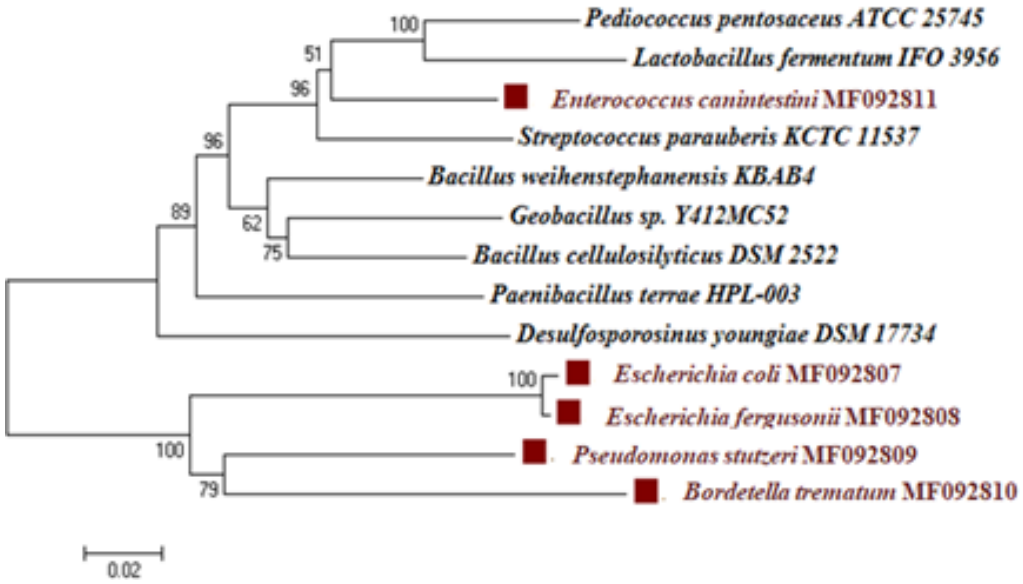

Fig. 2. The phylogenetic tree of bacterial cultures of consortium RV2 was constructed using the neighbour-joining method with aid of MEGA 6.0 program.

conventional treatment technologies and processes [23]. These microorganisms, largely found within the soil vicinity of textile and dye manufacturing industrial area, acclimatize themselves over the long time period in the presence of complex dyes due to their persistence in their microenvironment [24].

The bacterial culture composition of potent consortium RV2 studied through 16S rRNA analysis revealed that five bacterial species constitutes the consortium namely, *Enterococcus canintestini*, *E. coli* strain, *Escherichia fergusonii*, *Pseudomonas stutzeri* and *Bordetella trematum* representing two bacterial phyla Firmicutes and Proteobacteria respectively. Bacterial species from both these phyla are often reported possessing dye decolorizing property [25–27]. Fig. 2 shows the phylogenetic relationship amongst these species.

3.1. Influence of oxygen availability on Reactive Red 31 decolorization

The results of RR31 decolorization, clearly indicated that consortium RV2 under microaerophilic (static) condition decolorized (87.05% of 100 mg/L) Reactive Red 31 within 12 h in MSM medium at 37°C (Fig. 3). Under oxygen-rich conditions (at 120 rpm), consortium RV2 was incapable for metabolizing Reactive Red 31 and only 39.74% (of 100 mg/L) dye was decolorized in the similar time period.

It is well known that azoreductase enzyme based decolorization of azo dyes is a reductive mechanism and normally repressed in the presence of O_2 primarily because of oxygen scavenging competition between dye molecules and reduced electron carriers (e.g., NADH) [28]. Thus, higher

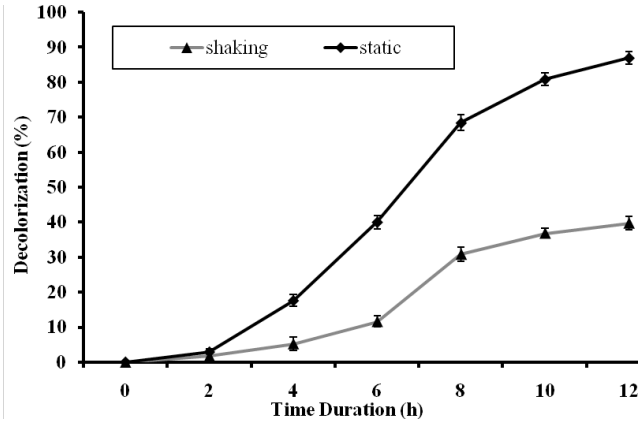

Fig. 3. Effect of aerobic (shaking at 120 rpm) and microaerophilic (static) conditions on decolorization of Reactive Red 31 (100 mg/L) by consortium RV2 at 37°C, pH 8.0.

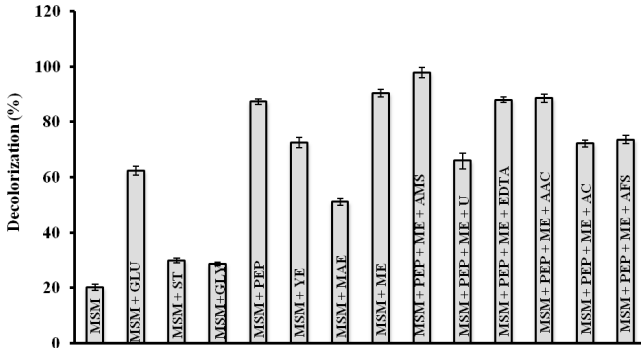

Fig. 4. Effect of co-substrates (supplemented 1 g/L for each substrate) on decolorization of Reactive Red 31 (100 mg/L) by the consortium RV2 under microaerophilic conditions, at 37°C: GLU-Glucose; ST-Starch; GLY-Glycerol; PEP-Peptone; YE-Yeast extract; MAE-Malt extract; ME-Meat extract; U-Urea; EDTA-Ethylene diamine tetraacetic acid; AAC-Ammonium acetate; AC-Ammonium chloride; AFS-Ammonium ferrous sulfate.

activities of azoreductase enzyme were usually detected under microaerophilic (static) conditions [29]. The study on Reactive Red 195 decolorization using an *E. coli* culture by Isik and Sponza [30] demonstrated similar results. Where, more than 72% of the dye was removed within 3 days under anaerobic conditions while at the same time 24 and 63% decolorization was observed under aerobic and microaerophilic incubations, respectively.

The azo dye decolorization by pure as well as mixed culture requires additional complex organic sources (i.e. supplementation of carbon or nitrogen sources) as reported by Lade et al. [31] and Saratale et al. [13]. Thus, the efficiency of consortium RV2 in decolorizing RR31 in the presence of various carbon and nitrogen sources was performed to determine their effects on decolorization.

Similarly, the study revealed that all supplementary inorganic co-substrates supported above 70% decolorization (except urea). But maximum decolorization was observed in the presence ammonium sulfate, peptone and meat extract (97.81) in 12 h (Fig. 4). In case of organic supplementations, meat extract (90.38%) followed by peptone (87.31%) showed the highest potential in terms of Reactive Red 31 decolorization. Thus, meat extract, peptone and ammonium sulfate was supplemented in MSM medium as carbon, nitrogen and energy source to obtain enhanced decolorization of Reactive Red 31 (97.81%). Similar results were reported by Modi et al. [32] where peptone was the best co-metabolite for efficient decolorization of Reactive Red 195 by *Bacillus cereus*. Furthermore, the addition of dextrose as additional carbon source decreases the % decolorization (62.35%) of Reactive Red 31. The negative effect of carbon sources like glucose on microaerophilic decolorization of dye has been described either due to a decrease in pH by acid formation or because of catabolic repression [33]. In a similar study carried out by Kalyani et al. [20] for the degradation Reactive Red 2 by *Pseudomonas sp*. SUK1, microaerophilic conditions showed better decolorization ability when compared to aerobic environment. Thus, further decolorization study of dye and effluent was carried out in microaerophilic (static) conditions only.

3.2. Effect of physico-chemical parameters on RR31 decolorization

Determination of optimum pH is a very crucial factor in dye decolorization process, as transport of dye molecule into the cell is pH dependent and thought to be rate limiting step for decolorization process [34]. Consortium RV2 was able to decolorize Reactive Red 31 dye at a broad range of pH, however, optimum pH for dye decolorization was found to be 8.0 (Fig. 5). The decrease in % decolorization was observed at lower pH (4–7) as well as higher pH (9–10). An incubation temperature of 37°C was found to be optimum for enhanced degradation of dye Reactive Red 31 by using consortium RV2 (Fig. 5). Further increase or decrease in the temperature decreased the extent of dye decolorization. Meanwhile, previous studies showed that extreme environmental conditions such as high or low temperatures would significantly inhibit decolorization [35]. Chakraborty et al. [36] reported that in degradative decolorization, the optimum temperature is necessary for the optimal activity of dye degradative enzymes. Decolorization performance (Fig. 5) at increasing dye concentrations (100–1000 mg/L) suggest its potential for decolorization (more than 98%) of 100–500 mg/L of Reactive Red 31. Waghmode et al. [37] reported the enhanced decolorization of dye Rubine GFL (50 mg/L within 30 h) using defined a consortium GG-BL of yeast and bacteria, *Galactomyces geotrichum* MTCC 1360 and *Brevibacillus laterosporus* MTCC 2298 respectively. The optimum pH and temperature for the decolorization of a sulfonated azo dye Reactive Orange 16 were 7–8 and 30–40°C, respectively using a *Bacillus sp*. [38].

3.3. Decolorization of different class of dyes by bacterial consortium RV2

Bacterial consortium RV2 had the ability to decolorize various reactive azo dyes with varying levels of efficiency. The differences observed in the decolorization might be explained by the structure and complexity of the dyes (Fig. 6). Owing to the fact that the monoazo dyes (RRRB,

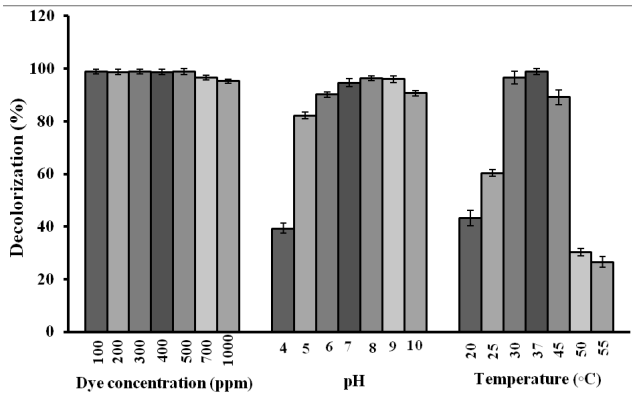

Fig. 5. Effect of dye concentration, pH, and temperature on decolorization of Reactive Red 31 (100 mg/L) by the consortium RV2 under microaerophilic conditions.

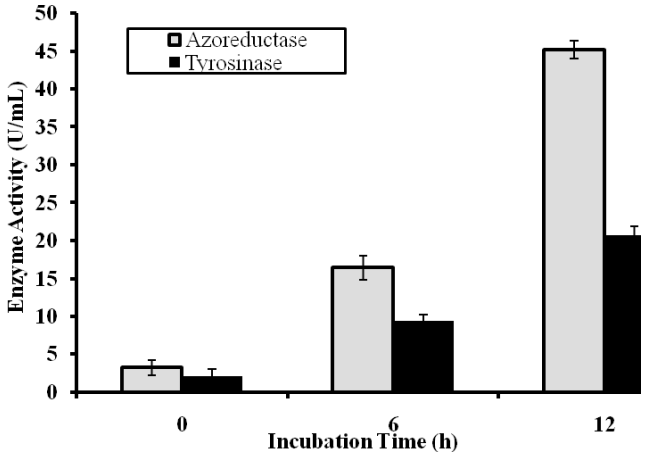

Fig. 7. Azoreductase and Tyrosinase activity in initial to final stage of Reactive red 31 decolorization at 37°C.

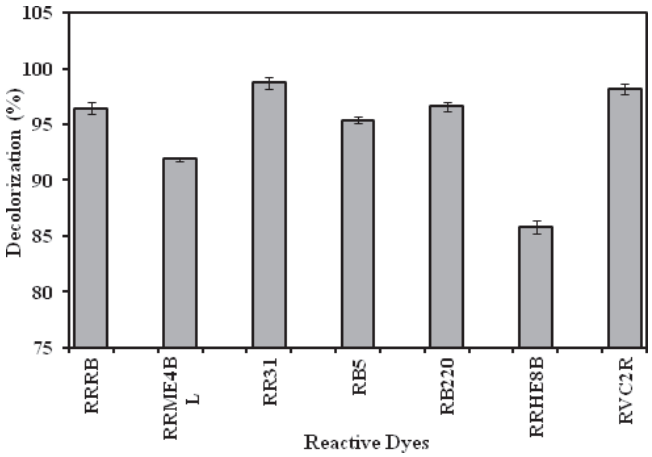

Fig. 6. Decolorization of different dyes (100 mg/L) by the bacterial consortium RV2 under microaerophilic conditions at 37°C, pH 8.0.

RRME4BL, RR31, RB5, RVC2R) possess simpler structures, they are more efficiently decolorized than diazo dyes (RB220, RRHE8B). This may be attributed to the lower activity of azoreductase against direct azo dyes [39].

3.4. Extracellular enzymatic studies

A major mechanism behind the bioremoval of different recalcitrant moieties in the microbial system is driven by the biotransformation enzymes [40–42]. To validate the mechanism of decolorization, enzyme activity was analyzed for azoreductase and tyrosinase. Enzyme activities were monitored after 12 h of incubation in bacterial consortium RV2 at the extracellular location. The higher levels of dye decolorization under static rather than shaking conditions suggest that azoreductase is playing an important role in cleavage of the N=N bond thereby leading to initiation of mineralization.

pH along with nitrogen and carbon supplementations are important physical parameters that influence the bioremediation of dye because they influence enzyme activity along with cell growth [43]. In the present study, a significant increase in enzyme activity of azoreductase and tyrosinase was observed after Reactive Red 31 decolorization by consortium RV2 (Fig. 7). The activity of azoreductase and tyrosinase in consortium after decolorization (45.23 U/mL and 20.69 U/mL respectively) was about 14 and 10 fold more than before decolorization (3.24 U/mL and 2.07 U/mL respectively) suggesting that both the enzymes might be induced by the addition of Reactive Red 31 dye, and thus results in a higher decolorization. The enzymatic activity indicates the contribution of oxidoreductive enzymes for the breakdown of Reactive Red 31 dye molecule into simple metabolites. Similarly, induction in the azo-reductase enzymes of *Galactomyces geotrichum* was reported by Jadhav et al. [44] for the decolorization of Methyl Red dye. From these results, we anticipated that the Reactive Red 31 decolorization was carried out via the enzymatic mechanism.

3.5. Screening of significant variables with statistical optimization study

3.5.1. Plackett–Burman design

In the first step of optimization, the influence of 11 medium components on the RR31 decolorization using bacterial consortium RV2 was investigated using the Plackett–Burman design (Table 1). Statistical analysis (Table 3) indicated that peptone (C), meat extract (D), pH (H) and dye concentration (L) were significant (p-values less than the significance level of 0.1), while other factors including KH_2PO_4 (A), $(NH_4)_2SO_4$ (B), $MgSO_4$ (E), NaCl (F), $CaCl_2$ (G), temperature (I) and inoculum size (J) were found to be insignificant due to p-values above the significance level.

The multiple correlation coefficients (R2) of this first-order model (0.93%) reveals a good correlation between observed and predicted response. For a good statistical model, the R2 value should be close to 1.0 where a value > 0.75 indicates the aptness of the model. Also, the model indicated that the predicted R2 value of 0.75 was in reasonable agreement with the adjusted R2 value of 0.69.

pH with Prob > F-value of 0.0032 was found to be the most influencing factor followed by concentrations of

Table 2
Plackett–Burman experimental design for screening significant variables affecting % decolorization of Reactive Red 31 dye [a]

Runs	A	B	C	D	E	F	G	H	J	K	L	Average % Decolorization[b]
1	0.75	1	0	0	0.25	10	0.01	9	37	3	500	5.36
2	0.25	1	0	1.25	0.75	2.5	0.03	9	37	3	100	78.36
3	0.25	1	1.5	0	0.75	10	0.03	5	26	3	500	10.89
4	0.75	0.25	1.5	1.25	0.75	2.5	0.01	5	37	3	500	15.23
5	0.25	1	1.5	1.25	0.25	2.5	0.01	9	26	5	500	99.89
6	0.75	1	0	1.25	0.75	10	0.01	5	26	5	100	11.25
7	0.75	0.25	0	0	0.75	2.5	0.03	9	26	5	500	3.69
8	0.25	0.25	0	1.25	0.25	10	0.03	5	37	5	500	13.69
9	0.75	0.25	1.5	1.25	0.25	10	0.03	9	26	3	100	99.87
10	0.75	1	1.5	0	0.25	2.5	0.03	5	37	5	100	12.35
11	0.5	0.625	0.75	0.625	0.5	6.25	0.02	7	31.5	4	300	74.56
12	0.25	0.25	0	0	0.25	2.5	0.01	5	26	3	100	5.68
13	0.25	0.25	1.5	0	0.75	10	0.01	9	37	5	100	99.67

[a]All the units were (g/L)
[b]The values were the mean values of triplicates.

Table 3
Analysis of variance for Plackett – Burman factorial model

Source	Degrees of Freedom	Sum of Squares	F-value	Prob>F	
Model	9	19541.45	80.49	0.0123	significant
KH_2PO_4	1	2144.82	79.51	0.0123	
$(NH_4)_2SO_4$	1	32.44	1.20	0.3872	
Peptone	1	4028.57	149.35	0.0066	
Meat extract	1	2719.54	100.82	0.0098	
$MgSO_4$	1	26.26	0.021	0.9078	
NaCl	1	54.32	2.01	0.2917	
$CaCl_2$	1	27.69	0.022	0.9053	
pH	1	8413.76	311.91	0.0032	
Temperature	1	3.64	0.13	0.7486	
Inoculum size	1	52.71	1.95	0.2970	
Dye concentration	1	2091.67	77.54	0.0127	
Curvature	1	1234.21	45.75	0.0212	
Residual error	2	53.95			
Cor Total	12	20829.61			

peptone (0.0066), meat Extract (0.0098), K_2HPO_4 (0.0123) and dye concentration (0.0127) in the medium (Table 2). Out of the five significant variables identified, K_2HPO_4 and dye concentration were exerting negative influence while the other factors such as pH, peptone and meat extract exerted a positive influence on % Decolorization of RR31 as indicated by the effect estimates (Table 1). Among these factors, the coefficient of the pH is 26.48, greater than that of any other parameters, which means that the pH had the most significant positive influence on decolorization. It meant that a relatively high pH might be needed for decolorization of Reactive Red 31 by the bacterial consortium.

3.5.2. Box-Behnken design

Based on the results of the Plackett–Burman design, four variables, including peptone (C), Meat Extract (D), pH (H) and dye concentration (L), were found to have a greater influence on Reactive Red 31 decolorization by the consortium RV2. Thereafter, the Box Behnken design was employed to optimize the level of the selected variables (Table 4).

The main goal of the experimental design was to determine the optimum values of the selected responses (% decolorization, azoreductase activity and tyrosinase activity) that result in the optimization of the responses. The optimum conditions found from the optimization search using Box-Behnken design are listed in Table 5.

Thus, the optimum values obtained after incubation from the statistical analysis with a Box-Behnken design with four design factors and three responses were: Average percent decolorization 99.24, azoreductase activity 45.23 U/mL and tyrosinase activity 20.69 U/mL.

The adequacy of the model was evaluated by coefficient of determination (R^2) and model p-value. Analysis of variance (ANOVA) of responses for average % decolorization, the rate of decolorization, azoreductase and tyrosinase assay with bacterial consortium RV2 were used to ensure a good model. The ANOVA analysis was required to justify the significance and adequacy of the model (Table 6). Prob > F less than 0.05 indicates model terms are significant.

The final responses for average % decolorization, azoreductase assay, tyrosinase assay terms of coded factors are given in Eqs. (3)–(5).

Table 4
Coded and actual values of the variables used in the Box-Behnken design

Independent variables	Codes	Level		
		–1	0	1
Peptone	A	0	1	2
Meat extract	B	0	1	2
pH	C	6.5	8.5	10.5
Dye concentration	D	100	300	500

$$\text{Average \% decolorization} = +9.68 + 1.32 * A + 0.71 * B + 0.55 * C - 0.15 * D - 1.61 * AB + 0.034 * AC - 0.065 * AD - 0.095 * BC - 0.13 * BD - 0.10 * CD - 0.76 * A^2 - 0.46 * B^2 - 1.45 * C^2 + 0.35 * D^2 \quad (3)$$

$$\text{Azo-reductase activity} = +5.14 + 1.30 * A + 0.56 * B + 0.28 * C + 0.23 * D - 0.94 * AB + 0.35 * AC - 0.17 * AD + 0.095 * BC - 0.067 * BD + 0.29 * CD - 0.42 * A^2 - 0.20 * B^2 - 1.55 * C^2 + 0.20 * D^2 \quad (4)$$

$$\text{Tyrosinase activity} = +3.44 + 0.78 * A + 0.34 * B + 0.23 * C + 0.14 * D - 0.66 * AB + 0.026 * AC - 0.092 * AD + 0.21 * BC + 2.498E - 003 * BD + 0.17 * CD - 0.18 * A^2 - 0.075 * B^2 - 0.86 * C^2 + 0.029 * D^2 \quad (5)$$

Table 5
Box-Behnken design in various runs along with Average % decolorization, azoreductase and tyrosinase activity as the responses

Run order	Independent variables				Average % decolorization	Azoreductase activity	Tyrosinase activity
	A	B	C	D			
1	0	–1	0	1	94.23	26.35	12.56
2	–1	0	–1	0	59.32	6.23	3.54
3	–1	0	1	0	66.47	5.23	5.47
4	0	–1	–1	0	65.23	10.26	6.34
5	–1	1	0	0	95.68	19.36	9.63
6	0	0	0	0	92.35	25.63	11.36
7	0	0	0	0	95.63	26.35	12.87
8	0	1	1	0	84.23	16.35	9.87
9	0	1	0	1	96.35	32.25	14.56
10	0	–1	1	0	76.23	11.23	5.91
11	0	0	–1	1	55.36	9.36	4.23
12	–1	0	0	1	92.36	19.68	8.69
13	0	1	–1	0	74.23	12.36	5.68
14	0	0	1	1	88.63	20.36	8.69
15	1	0	0	–1	99.35	36.25	16.36
16	0	0	–1	–1	65.47	12.36	5.78
17	1	–1	0	0	99.24	41.23	19.23
18	1	0	–1	0	69.24	10.25	5.69
19	1	1	0	0	99.88	45.23	20.69
20	0	–1	0	–1	88.75	18.65	8.67
21	1	0	1	0	92.68	19.23	8.69
22	–1	–1	0	0	9.25	0.1	0.09
23	0	1	0	–1	96.37	26.35	10.28
24	–1	0	0	–1	91.23	12.36	6.33
25	0	0	1	–1	75.36	14.56	6.87
26	0	0	0	0	98.57	27.36	11.25
27	1	0	0	1	99.67	39.25	16.89

Table 6
ANOVA analysis for average percent decolorization, azoreductase and tyrosinase assay

Source	DF	Average % decolorization			Azoreductase			Tyrosinase		
		Sum of squares	F value	Prob>F	Sum of squares	F value	Prob>F	Sum of squares	F value	Prob>F
Model	14	58.48	4.33	0.0076	46.32	6.07	0.0017	16.24	3.56	0.0168
A	1	20.93	21.67	0.0006	20.20	37.08	<0.0001	7.26	22.25	0.0005
B	1	6.13	6.35	0.0269	3.8	6.98	0.0215	1.38	4.24	0.0618
C	1	3.64	3.77	0.0759	0.97	1.77	0.2079	0.65	2.00	0.1826
D	1	0.27	0.28	0.6067	0.64	1.17	0.3007	0.24	0.73	0.4110
AB	1	10.32	10.69	0.0067	3.57	6.56	0.0250	1.74	5.34	0.0394
AC	1	4.506E	4.666E-003	0.9467	0.48	0.89	0.3640	2.766E-003	8.479E-003	0.9282
AD	1	0.017	0.018	0.8965	0.11	0.21	0.6550	0.034	0.10	0.7536
BC	1	0.036	0.037	0.8507	0.036	0.066	0.8013	0.18	0.55	0.4736
BD	1	0.073	0.075	0.7886	0.018	0.033	0.8584	2.495E-005	7.648E-005	0.9932
CD	1	0.043	0.045	0.8355	0.33	0.61	0.4500	0.11	0.35	0.5660
A^2	1	3.09	3.20	0.0990	0.93	1.72	0.2147	0.17	0.51	0.4883
B^2	1	1.14	1.18	0.2989	0.21	0.38	0.5484	0.030	0.093	0.7660
C^2	1	11.24	11.64	0.0052	12.88	23.65	0.0004	3.93	12.04	0.0046
D^2	1	0.67	0.69	0.4228	0.21	0.38	0.5466	4.597E-003	0.014	0.9075
Residual Error	12	11.59			6.54			3.92		
Lack of Fit	10	11.58	357.55		6.52	91.57	0.0108	3.88	22.87	0.0426
Pure Error	2	6.479E-			0.014			0.034		
Cor Total	26	70.07			52.86			20.16		
C.V.			11.37			17.30			19.32	
R^2			0.8346			0.8764			0.8058	
Adj R^2			0.6417			0.7321			0.5792	

Fig. 8 shows the interactive effect between pH and meat extract. The average percent decolorization, azoreductase and tyrosinase activity were found to be increasing with the increase in meat extract concentration in the pH range of 6.5–8.5. Beyond this level of pH, the concentration of yeast extract failed to significantly enhance the decolorization and enzyme production. The interaction between pH and meat extract concentration indicated that pH is having a profound effect on dye decolorization and enzyme production almost masking the effect of meat extract. According to previous reports, the optimal pH for most of the bacteria to decolorize dyes was at a neutral pH value or a slightly alkaline pH value [45]. This could be due to enhanced solubilization and transport of dye molecule across the cell membrane at slightly alkaline pH. However, the rate of decolorization was much lower at strongly acidic (6.5) and alkaline pH (10.5). These results corroborate earlier reports of Wang et al. [46] and Agrawal et al. [47].

The interactive influence of peptone and dye concentration on average percent decolorization, azoreductase and tyrosinase activity is illustrated in Fig. 8. At lower concentrations of peptone, no significant changes in decolorization and enzyme production were observed regardless of the dye concentration. With the peptone concentration increase in decolorization and enzyme production was observed, while no significant change was observed with increase in dye concentration. The effect of various nitrogen sources including peptone on enzymes productivity of various organisms found to be greatly controversial [48]. Although it was established that the enzyme production by *Fusarium incarnatum* LD-3, *Pleurotus ostreatus* and *Phanerochaete chrysosporium* was enhanced by nitrogen limitation [49–51], there are reports that peptone supplemented media resulted in enhanced production of enzymes in *Trametes villosa* and *Paraconiothyrium variabile* [52,53].

The enzymatic mechanism for biodecolorization and biodegradation acts upon oxidoreductases and included both bacterial azoreductase and tyrosinase [54]. In the initial stage, azo bond was cleaved by azo reductase using two equivalents of NADH to reduce one equivalent of the azo compound into two equivalents of aniline product. Subsequently, the intermediates such as aromatic amines result in degradation by various oxidative enzymes that broke aromatic compounds [55,56].

4. Conclusion

Bacterial consortium RV2 possesses the ability for the effective decolorization of Reactive Red 31 dye. Enhanced dye decolorization in microaerophilic as compared to agitate conditions supports the involvement of azoreductase and tyrosinase in decolorization mechanism. A significant increase in azoreductase and tyrosinase activities in the culture broth were obtained after complete decolorization

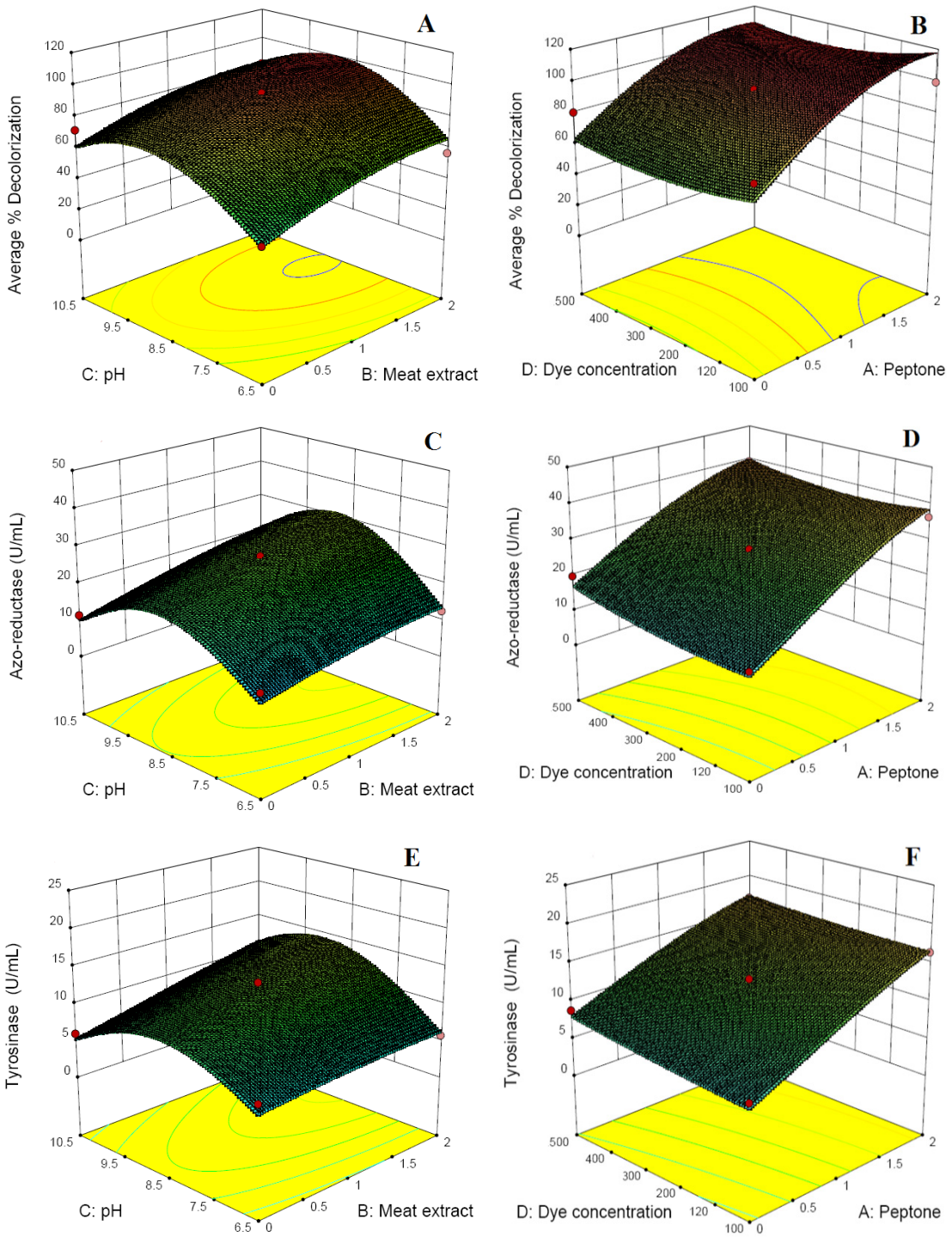

Fig. 8. Response surface for average percent decolorization, azoreductase and tyrosinase production. The three-dimensional plot shows the influence of pH, peptone, meat extract and dye concentration on dye decolorization.

as compared to initial stage suggests induction of enzymes during the process. The Plackett-Burman and Box-Benkhen Design were successfully applied for the rapid screening and optimizing the operational requirements for RR31 decolorization, azoreductase and tyrosinase production. The optimization of various cultural and nutritional parameters for the Reactive Red 31 decolorization by bacterial consortium showed that the decolorization and enzyme production by consortium RV2 is governed by parameters such as pH, peptone, meat extract and dye concentration. Optimized levels of peptone, meat extract, and pH were found to be 2.0 g/L, 2.0 g/L and 8.5 respectively.

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