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Sustainable biodegradation of textile dye reactive blue 222 by the novel strain *Enterobacter* CU2004, isolated from the industrial waste: A design of experiment based optimization study and characterisation of metabolites

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ABSTRACT

Reactive Blue 222 (RB222) is widely used in textile industries and hence a common recalcitrant pollutant in the industrial effluent. Bioremediation of this dye is of significance as its one of the complex dyes with high molecular weight. In the present study, we isolated a novel bacterial strain *Enterobacter* CU2004 from the industrial waste and characterize using16S rRNA gene sequencing. Its potential to dye degradation was evaluated in a simple minimal salt media with the parameters namely dye concentration (100–1000 ppm), pH (4–9), temperature (15–55°C), Carbon source (Lactose, Sucrose, Glucose, Starch, and Fructose), and Nitrogen source (Casein, Yeast extract, Peptone, Tryptone, Ammonium sulphate, and Urea) in a 24 h culture. Finally, data obtained were extended to design of experiment based optimization for the degradation efficacy of *Enterobacter* CU2004 and to validated design space was established. The novelty is in optimizing the design space parameters for highest percentage of degradation ≥90% by the bacterial isolate *Enterobacter* CU2004 were finalized as 30–37°C temperature, 133–249 ppm dye concentration, Lactose as Carbon source, Yeast extract as Nitrogen source, and the pH as 8. Microbial dye degradation was confirmed by FTIR, HPLC and GCMS studies. Further studies revealed the dye intermediates and the potential of *Enterobacter CU2004* toward the degradation of complex, high molecular weight industrial dye RB222.

1. INTRODUCTION

Anthropogenic activities of humans are adversely affecting the environment. One such activity is the mass production and utilization of synthetic dyes [1]. Various industries use synthetic dyes as raw materials and release the unused ones into industrial effluents. Industries such as printing, pharmaceutical, food, and textile are the major consumers of these dyes [2]. As the demand for these dyes increases, their production has also increased over the past few decades. In 2020, India produced 191 thousand metric tons of dyes and is considered one of the leading producers manufacturing 80.000 products [3]. Nearly 10,000 dyes and pigments are now available for textile industries [4]. Textile industries are the leading consumers of these dyes and release unused dyes frequently at a 15% concentration into water bodies [5-7] Globally, it is estimated that approximately 280,000 tons of textile dyes are discharged into water bodies annually in the untreated form [8,9]. Removal and degradation of these dyes in the industrial effluent is one of the major environmental challenges and therefore, gaining more attention over the past few decades [10,11]. Even at a lower concentration of 1 ppm, these dyes can affect the nature and ecosystem of water. These dyes can block sunlight penetration and affect the photosynthetic process of aquatic plants [12]. Furthermore, it can lead to low dissolved oxygen levels and an increase in pH, BOD, and COD of water [13,14]. Industrial dyes are recalcitrant xenobiotics and thus tend to persist for a long time in the environment. Studies have also proved that they seriously affect all living forms as they are carcinogens, mutagens and teratogens. When humans are continuously exposed to these dyes, it can lead to health issues such as bladder cancer and skin allergy [15,16].

Among the industrial dyes, reactive dyes are most extensively used as they make the staining process is simple and cost-effective. These dyes contain reactive groups that can form a covalent bond with the fabric materials and thus resist wash fastness and lightfastness. Reactive

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dyes are used in dying of cellulose and polyamide materials in textile industries and, this makes reactive dyes as the one of the most common pollutants in industrial effluent [17-19]. Reactive dyes exhibit high solubility in water with varying complex chemical structures such as reactive blue 19 and reactive black 5 [20].

Microbial bioremediation is widely accepted as they catalyze degradation and complete mineralization of dyes at a faster rate than the conventional methods [21,22]. However, physiochemical methods are expensive, less efficient, and produce secondary sludge. Physiochemical methods such as biosorption by dead biomass of *Rhizopus arrhizus* [23] and basidiomycetes immobilized in *Luffa cylindricall* for RB222 were not much effective as they relied on passive adsorption principle rather than degradation of dyes [18]. *Enterobacters,* are gram-negative and natural inhabitant of the digestive tract in warm-blooded animals, are the most prevalent microbes in sewage waste. These are facultative microbes capable of degrading dyes at aerobic and anaerobic conditions [24,25]. Another significant feature of this isolate is forming a natural biofilm, enhancing its biodegradation ability. This natural biofilm can degrade higher concentrations of dyes and withstand adverse conditions otherwise not possible by discrete microbes [26]. Hence, *Enterobacter* sps may be considered as the most feasible and eco-friendly bio-remediator for reactive dye biotransformation to nontoxic end products [27,28].

The dye degradation capabilities of *Enterobacter* sps were reported elsewhere, majorly for Azo dyes [29]. Nevertheless, reports on applying this bacterial strain for bioremediation of Reactive Blue dye 222 are not reported till date to the best of our knowledge. RB222 is one of the most common dyes used in Indian textile industries. It is a highly recalcitrant dye due to its high molecular weight (1357.49 g mol^{-1}) and further complicated and complex with two dia azo-groups which poses a problem in its uptake by the microbe bioremediation. Although other chemical methods are available for the degradation of this dye; however, due to its structural complexity, the microbial degradation of RB222 is challenging [30,31]. The key advantages of using this novel isolate are its ability to form biofilm and hence can resist and thrive at adverse condition and facilitates complete mineralization of RB222. Furthermore, this isolate can make the entire process of bioremediation of RB222 in industries as economical and eco-friendly since *Enterobacter* sp. CU2004 are facultative anaerobes showed its best performance under static conditions without any need for aeration or agitation. The isolate was able to metabolite RB222 dye in simple media without carbon or nitrogen source; however, their efficiency improved with cometabolism of carbon and nitrogen source.

Present work emphasis, the novelty in optimizing various physical and chemical parameters (pH, temperature, agitation, carbon, and nitrogen sources) through multivariate design of experiments (DOE) for the new isolate *Enterobacter* sp. CU2004 from the textile industrial waste to completely mineralize the RB222. Studies provide design space and knowledge on dye degradation pathway.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Reactive Blue 222 (RB222) dye (Praasa Overseas, India), minimal salt media (MSM) broth (M1253) comprising of Sodium chloride (2.5 g l^{-1}), Disodium phosphate (33.5 g L^{-1}), Potassium phosphate (15 g L^{-1}) and Ammonium chloride (5 g L^{-1}) used in dye degradation studies was procured from Hi-Media Laboratories (Mumbai, India), peptone, yeast extract, tryptone, and agar (Bacteriological grade, Himedia, India). Glucose, sucrose, lactose, fructose, starch, urea, ammonium sulfate, methanol, and ethyl acetate chemicals were from purchased from SRL Ltd., India.

2.2. Isolation and Screening of the Microorganisms

Waste water sludge (collected from Peenya industrial area, Bangalore, India) was inoculated into the MSM (10% v/v) and incubated with 100 ppm RB222 on an orbital shaker at 37°C, 120 rpm for 24 h. Further sub-culturing was performed at a high concentration of dye (200 ppm) with similar conditions and inoculum strength (10%). The final subculture was performed with 500 ppm dye. Bacterial strains capable of utilizing the dye were screened by serial dilution and spread plated on MSM agar supplemented with 100 ppm dye. We observed five colonies with prominent dye degradation properties and maintained them as a pure culture on MSM agar with 50 ppm dye for further studies. These isolated bacterial strains were cultured with 100 ppm dye concentration in MSM broth and incubated in an orbital shaker at 37°C, 120 rpm for 24 h. Cultures were centrifuged at 8000 rpm for 5 min, and absorbance of the supernatant was measured at 615 nm using UV–Visible spectrophotometer (Shimadzu UV).

Percentage of degradation by each isolate was estimated using the formula [32]

$$\%PDD = \frac{Ac - Ai}{Ac} \times 100$$

Percentage Dye Degradation = %PDD

Ac (Absorbance control): Media + Dye (100 ppm)

Ai (Absorbance inoculated): Media + Dye (100 ppm) + Bacteria

Bacterial isolate (B1) with the highest decolourization efficiency was selected for further studies.

2.3. Identification of Bacterial Isolate

For identification of the best performing bacterial isolate B1, it was outsourced to Barcode Biosciences, Bangalore, India. Purified genomic DNA of the bacterial isolate was subjected to 16S rRNA sequencing using BDT v3.1 kit and ABI 3730 × l Genetic Analyzer through Sanger's di-deoxy chain termination method. Finally, the bacterial strain was identified by using Bioinformatics tools, namely, NCBI BLAST and MEGA X [33]. The model of sequence evolution used was a maximum composite likelihood, and for rate variation, the gamma distribution of 1 was used.

2.4. Univariate Study

Univariate experiments one factor at a time (OFAT) were initially performed to assess the dye degradation potential of the identified isolate (B1) at different physical and chemical conditions in an aqueous medium. Various experimental conditions such as pH (4–9), temperature (16–55°C), inoculum size (0.5–4% w/v), dye concentrations (100–1000 ppm), 1% w/v Carbon (glucose, sucrose, lactose, fructose, and starch), and nitrogen (casein, yeast extract, tryptone, peptone, urea, and ammonium sulfate) sources were maintained in individual sets in liquid culture media (MSM broth). Cultures were incubated at 37°C under static conditions except for temperature and aeration studies. PDD was calculated as per the formula mentioned earlier. All the experiments were performed as triplicates, and the results are expressed as mean ± SE (Standard error). The data were analyzed statistically by One-way analysis of variance (ANOVA) (IBM SPSS Statistics software V21.0). The statistical significance level was set as $P \leq 0.05$.

2.5. Multivariate Study DOE

A multivariate and DOE approach as per earlier studies [34] was considered to optimize the bacterial isolate mediated % degradation of the dye. DOE is a tool to optimize desirable process performance based on a robust and multivariate statistical methodology. In this study, based on univariate experiment results, five major variables namely temperature (25–37°C), RB222 concentration (100–250 ppm), pH (5–8), Carbon (Control and Lactose), and Nitrogen (Control and Yeast extract) sources were selected. A customized quadratic response surface model was adopted for this study and the experiments were conducted with 29 random runs, as obtained by the DOE software (Design-Expert 12). The experimental design is shown in Table 1. Finally, a design space was established and validated with post-process optimization run.

2.6. Dye Degradation Studies

Dye metabolites was concentrated from inoculation broth using ethyl acetate solvent at different intervals of incubation period. Organic layer was subjected to rotatory evaporation to obtain crystals of dye intermediate metabolite for FTIR, HPLC, and GCMS studies. FTIR analysis was performed in mid-IR range (400–4000 cm^{-1}) using Shimadzu-IrSpirit 1800 with ATR accessory. FTIR peaks obtained for dye intermediate metabolites due to the action of bacteria was compared with the parental dye spectrum.

High-performance liquid chromatography (Shimadzu-LCMS 8040) was performed to examine the dye degradation with 50% methanol and 50% water as mobile phase and analytes were separated in C 18 column of length 4.6 mm with run rate of 1 mL/min. To examine the dye degradation UV detector was set at λ max of RB-222 (615 nm), and dye degraded metabolites (280 nm), respectively.

Gas chromatography-mass spectroscopy was performed to determine the metabolites produced in the course of microbial degradation of RB222 using GCMS-QP2010 model. A sample volume of 1 µL was injected and the injector temperature was maintained at 280°C. Helium was the carrier gas, flown at 1 mL min^{-1}. The temperature of the column was set at 60°C held for 4 min, and successively increased to 200°C at a ramp rate of 10°C min^{-1}, holding 3 min, followed by 200°C–280°C at a ramp rate of 10°C min^{-1}, holding the temperature for 9 min. Mass spectra were obtained at 70 eV. GC–MS spectra were analyzed using the software Shimadzu post-run analysis.

Table 1: Experiment details for the design of experiment setup with results (% Decolorization).

Run	Temperature (°C)	Dye concentration (ppm)	pH	Carbon source	Nitrogen source	Decolorization (%)
1	28.48	202.75	7.40	Lactose	Yeast extract	92
2	25.00	250.00	8.00	Lactose	Control	32
3	26.50	185.50	5.00	Control	Yeast extract	66
4	26.92	100.00	5.00	Control	Control	34
5	32.56	152.50	8.00	Lactose	Control	59
6	28.30	100.00	7.63	Control	Yeast extract	70
7	25.00	100.00	8.00	Lactose	Control	63
8	25.00	250.00	5.00	Lactose	Control	50
9	37.00	100.00	7.48	Control	Control	29
10	25.00	250.00	8.00	Control	Yeast extract	77
11	32.14	100.00	6.43	Lactose	Yeast extract	64
12	37.00	100.00	8.00	Lactose	Yeast extract	81
13	28.30	100.00	7.63	Control	Yeast extract	88
14	37.00	104.67	5.38	Control	Yeast extract	41
15	25.00	100.00	5.54	Lactose	Yeast extract	48
16	33.46	148.00	5.62	Lactose	Control	71
17	37.00	250.00	7.48	Lactose	Control	83
18	33.46	148.00	5.62	control	Yeast extract	69
19	32.14	250.00	6.35	Control	Yeast extract	77
20	37.00	190.00	8.00	Control	Yeast extract	76
21	30.52	227.50	8.00	Control	Control	70
22	29.62	227.50	5.41	Lactose	Yeast extract	75
23	28.48	202.75	7.40	Lactose	Yeast extract	93
24	37.00	250.00	5.00	Lactose	Yeast extract	79
25	37.00	184.00	6.59	Lactose	Yeast extract	84
26	32.14	250.00	6.35	Control	Yeast extract	80
27	37.00	250.00	5.00	Control	Control	42
28	25.00	181.75	6.56	Control	Control	60
29	25.00	181.75	6.56	Control	Control	61

2.7. Statistical Analysis

The data obtained from dye degradation studies were tested for statistical significance by comparing the means of different test conditions using one-way ANOVA. The data were considered significant if $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Isolation and Screening of Microorganisms

From preliminary screening, five different strains of bacteria on the solid MSM medium and tested for their degradation potential against 100 ppm of RB222 dye by using the enrichment method as performed by [1]. After 24 h of incubation in the MSM broth, isolate B1 showed the highest dye degradation activity. Based on this data, B1 isolate was selected for further studies.

The phylogenetic tree was constructed using MEGA X software [35] to compare the isolate B1 with relevant bacteria. The new isolate showed 99% homology with *Enterobacter sp. strain* DMKU-RP206 and is considered a new branch in the phylogenetic tree of genus *Enterobacter* [Figure 1]. 16S rRNA sequencing coupled with Neighbour-Joining algorithm is a common methodology used in the literature to identify new species [36]. Previously isolated two bacterial strains capable of degrading malachite green and crystal violet were confirmed with 99% similarity to *Enterobacter* sp HSL99 and *Enterobacter* sp HSL69, respectively. Isolate RKS13 capable of degrading methylene blue revealed the closest similarity with *Bacillus albus* [13]. Similarly, 16S rRNA sequencing revealed that isolate T2 could decolorize reactive blue160. T2 exhibited 96% similarity to *Bacillus subtilis* [37]. In this study, NCBI BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search and Neighbour-Joining phylogenetic tree revealed that the newly isolated strain B1 as *Enterobacter s*p with 99% target sequence coverage and 100% bootstrap support. The stain name was designated as *Enterobacter* Sp. CU2004. The sequence was deposited to NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/, Accession No MF125281.1).

3.2. Bacterial Growth Kinetics and Dye Degradation

The batch culture was established with 100 ppm of RB222 dye in the MSM broth and incubated at 37°C under static conditions for 12 h with an inoculum size of 4% (v/v). Bacterial growth and dye degradation were monitored at a regular 2 h, and a growth kinetics graph was plotted [Figure 2]. It was evident from the plot that the optical density of cell suspension increased exponentially with the time when measured at 600 nm. Dye degradation also increased when examined at 615 nm at the same interval. The optical density of the cell was increased from 0.256 to 2.016, indicating microbial growth. Simultaneously degradation percentage of the dye also increased from 0.067 to 33% [38]. It showed in a similar study that degradation of methyl orange also significantly increased with the increase in optical density of *Bacillus stratosphericus* SCA1007 when examined during the incubation period of 0–12 h. Therefore, the result of our study established the positive correlation between microbial growth and dye degradation.

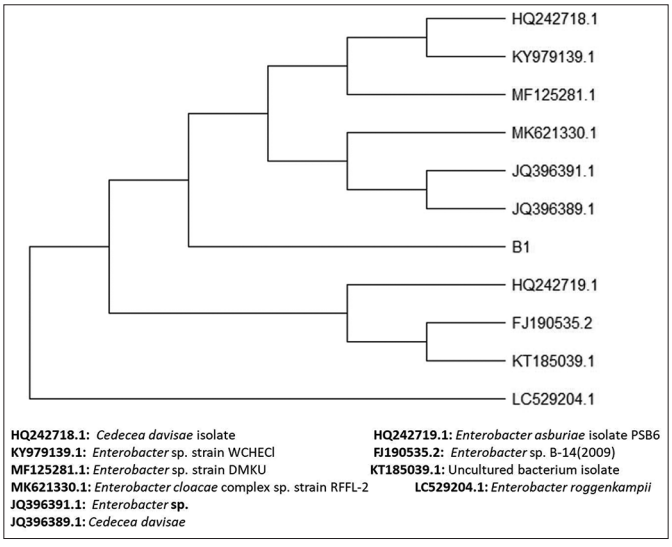

Figure 1: Phylogenetic tree of isolate *Enterobacter* CU2004 (B1) using Neighbor Joining method by MEGA X.

3.3. Univariate Study

For preliminary optimization of the dye decolorization efficiency of the isolate B1 (*Enterobacter* Sp. CU2004), we initially performed experiments by varying OFAT. Input parameters were pH, temperature (°C), inoculum size (%, v/v), dye concentration (ppm), and Carbon-Nitrogen sources. Hydrogen ion concentration in the medium, that is, pH, generally affects the decolorization ability of any bacterial isolate. Within the studied range [pH 4–9, Figure 3a], we observed that pH 7 gave the optimum efficacy for decolorization (41.24%). The beneficial effect of neutral pH on the decolorization of bacterial isolates was studied elsewhere. Ramlan *et al*. [39] reported optimum performance of *Brevibacillus panacihumi* ZBI decolorization of Reactive Blue 5 at neutral pH. Jirasripongpun *et al*. [40] also performed univariate studies and confirmed that *Enterobacter* Sp. exhibited its optimal decolorization of reactive red 195 at pH 7. Nevertheless, outcomes of these studies were limited by the fact that they were univariate in nature and effect of other parameters along with pH was not evaluated [41].

In the next set of the experiment, the effect of different temperatures (15–55°C) on the degradation potential of the isolate B1 was studied. Maximal degradation (81.81%) was recorded at 37°C [Figure 3b]. Further, higher temperature like 55°C negatively affected the degradation efficacy of B1 to as low as 3.81%. Higher temperature results in loss of cell viability, denaturation of vital enzymes, and disintegration of cellular membrane. The earlier study, Roy *et al*. (2020) [36] showed similar observation for *B. subtilis* for degradation of the dye reactive blue 172. *Enterobacter* sp. could effectively degrade

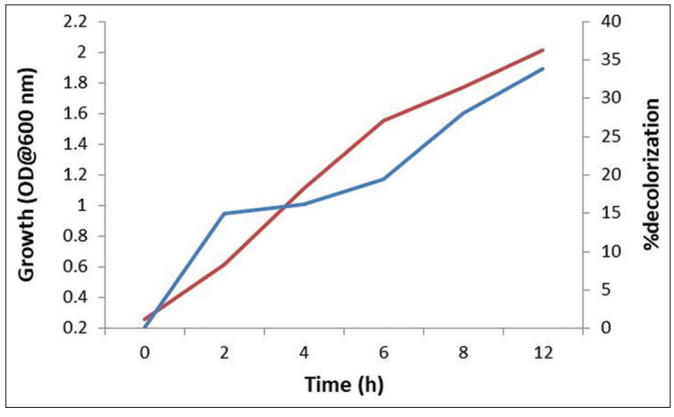

Figure 2: Growth kinetics of isolate *Enterobacter* CU2004 (Time vs. growth and percentage of dye degradation).

crystal violet at 35°C [42]. In general, microbes showed its promising dye degradation potential within the temperature range of 30–40°C.

Dye degradation was performed with varying inoculum sizes ranging from (0.5 to 4% v/v). Inoculum size had a direct effect on decolorization ability of isolate B1. Decolorization of RB222 dye at concentration of 100 ppm increased with increase in inoculum size [Figure 3f]. About 80% of dye decolorization was obtained with 4% (v/v) inoculum without adding carbon or nitrogen sources when incubated for 24 h. This confirms the active participation of B1 in the dye decolorization. A similar study reported improved degradation of direct blue 14 by *Bacillus fermus* (Kx898362) with the high inoculum size of 5% (v/v) [43].

The concentration of dye had a significant effect on dye decolorization ability of isolate. As the dye concentration was increased from 100 to 1000 ppm, the decolorization ability of the isolate B1 gradually reduced (81.51–51.53%) [Figure 3d]. Higher concentration of dye might pose a toxic effect on the microbial growth, metabolism, and saturating the active sites of the enzymes involved in dye decolorization, leading to reduced microbial efficacy [37]. noted that *Bacillus* sps could make maximum biofilm at low reactive red 170 dye concentration of 40 mg/ml. Mostly microbes failed to utilize azo dyes for their metabolism and growth efficiently. However, the results of the current study proved the isolate *Enterobacter* CU2005 could tolerate and decolorize RB222 at relatively higher concentration that that of the microorganisms reported in the literature.

Batch culture with 100 ppm of RB222 in MSM supplemented with different Carbon (Glucose, sucrose, lactose, fructose, and starch) and Nitrogen (Peptone, tryptone, caseine, yeast extract, urea, and ammonium sulfate) sources were set to study their effect on dye decolorization ability of the isolate B1. When the media was supplemented with carbon or nitrogen sources as cosubstrate, decolorization efficiency drastically increased. Lactose was found to be the ideal carbon source for showing higher percentage of dye decolorization (90%) when compared with the control, namely, without carbon source (81.5%) [Figure 3e]. Medium supplemented with yeast extract showed maximum decolorization of 69% among other nitrogen sources [Figure 3d]. Similar observations were reported elsewhere [44]. It showed an increase in the Remazol black B dye decolorization ability of *Bacillus* spp when the media was supplemented with the Carbon source Starch. Overall, the color removal ability of isolate B1 had greatly improved with the addition of organic carbon, while sole nitrogen supplements had less significance in the process.

3.4. DOE

DOE based multivariate experimental strategy is considered user-friendly, cost-effective, and can provide rapid results in a short span of time when compared with the conventional univariate or one-factor experimentation. Synergistic effect of a number of variables can be obtained from DOE based studies. Further, design space developed through DOE, can precisely provide effective parameter ranges to achieve expected output. The previous literature effectively utilized DOE strategy to optimize dye degradation [45,46]; however, application of such strategy for microorganism mediated dye degradation is scarce [47].

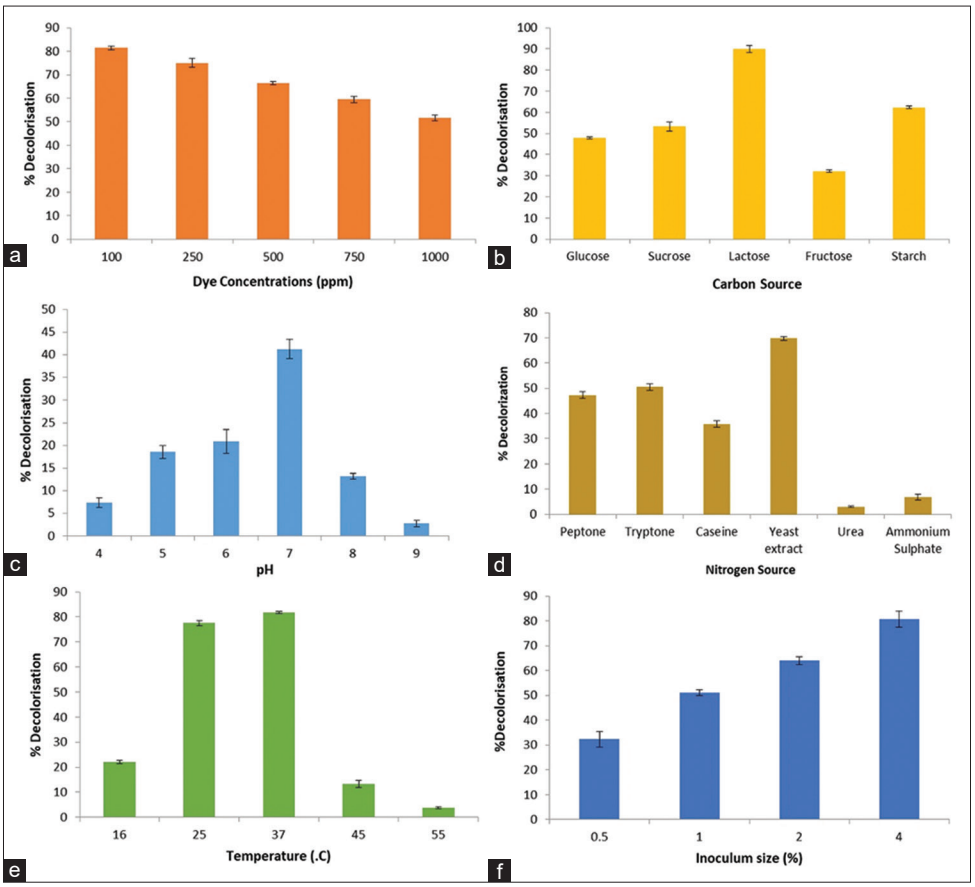

Figure 3: Graphical representation of Univariate experiments, (a) pH, (b) Temperature (°C), (c) Inoculum size (%), (d) Dye concentration (ppm), (e) Carbon source (1%) and (f) Nitrogen source (1%); All values are represented as mean ± SE; All values are statistically significant as performed by IBM SPSS software.

3.5. Selection of Model

The model F-value of 9.50 as determined by ANOVA implied that the model selected was statistically significant ($P < 0.00001$). There was only a 0.01% chance that an F-value this large could occur due to noise. The Lack of Fit F-value of 2.88 implied that it was not statistically significant relative to the pure error. There was a 12.50% chance that a lack of fit F-value this large could occur due to noise. Finally, the lack of fit was statistically insignificant ($P = 0.1250$). Based on this statistical analysis, a reduced quadratic model was selected. Model terms, namely, dye concentration (B), pH (C), carbon (D), and nitrogen (E) sources independently and, interactions of temperature X dye concentration (AB) and temperature X sugar (AD) were found statistically significant [Table 2]. Temperature X dye concentration (AB) was selected as interaction partners for further evaluation. The empirical relation between % degradation and factors was formed as: %degradation = 74.34 + 1.51A + 6.51B + 8.24C + 4.23D + 8.61E + 6.82AB + 6.68AD + 4.63CE + −8.48A2–8.57B2.

3.6. Comparative Contour Plots

Comparative two-dimensional contour plots [Figure 4] comprising the global range (29–93%) of the response (% degradation of dye) aided in

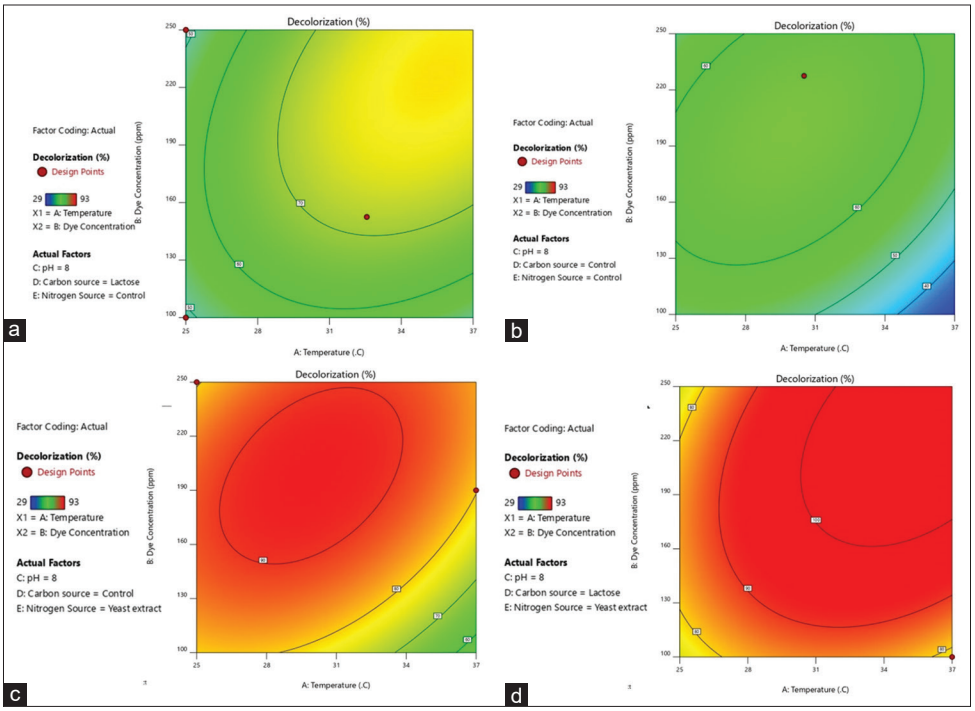

Figure 4: Two-dimensional contour plots of RB222 degradation by *Enterobacter* Sp. CU2004 as generated by design expert software for the process parameters (control, lactose, yeast extract and pH 8).

Table 2: ANOVA for the decolorization of RB222 by *Enterobacter* Sp. CU2004.

Source	Sum of squares	df	Mean square	F-value	*P*-value	Level of significance
Model	7595.22	10	759.52	9.50	<0.0001	Significant
A-Temperature	38.37	1	38.37	0.4801	0.4972	
B-Dye concentration	771.30	1	771.30	9.65	0.0061	Significant
C-pH	1078.33	1	1078.33	13.49	0.0017	Significant
D-Sugar	499.89	1	499.89	6.25	0.0223	Significant
E-Nitrogen source	1964.11	1	1964.11	24.57	0.0001	Significant
AB	515.18	1	515.18	6.45	0.0206	Significant
AD	698.75	1	698.75	8.74	0.0084	Significant
CE	340.33	1	340.33	4.26	0.0538	significant
A^2	353.04	1	353.04	4.42	0.0499	
B^2	350.08	1	350.08	4.38	0.0508	
Residual	1438.78	18	79.93			
Lack of fit	1269.28	13	97.64	2.88	0.1250	Not significant
Pure error	169.50	5	33.90			
Cor total	9034.00	28				

ANOVA: Analysis of variance

the selection of the other terms (D-Control/Lactose, E-Control/Yeast extract, and pH), along with the interaction term (AB). The result of the comparative contour plots indicated the higher decolorization rate in the lactose and yeast extract conditions when compared with the control at pH 8.

3.7. Establishment of Design Space and Finalizing Input Parameter Ranges

In this optimization experiment, we attempted to establish design space considering a cutoff of minimum 90% decolorization response and covering a maximum range of input parameters to achieve the response target. As shown in the Figure 5, design space parameters for the ≥90% degradation was established as 30–37°C temperature, 133–249 ppm dye concentration, lactose as carbon source, and yeast extract as nitrogen source, with the pH to be maintained at 8.

3.8. Validation of the Established Design Space

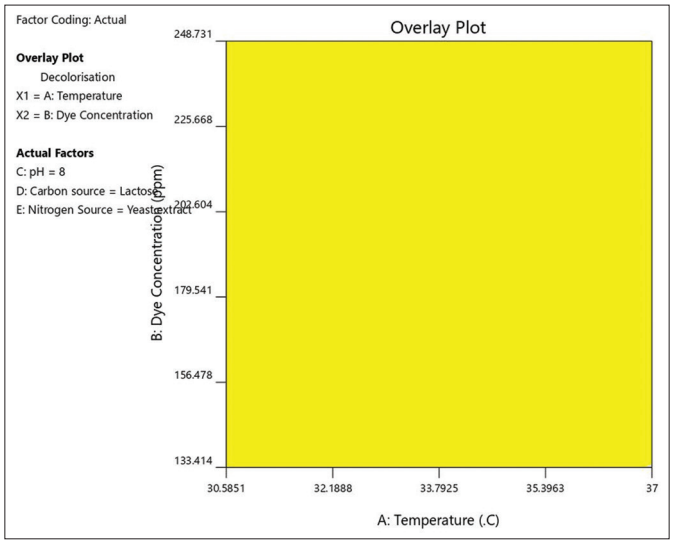

Figure 5: Design space for the optimized process parameters to degrade RB222 by *Enterobacter* Sp. CU2004.

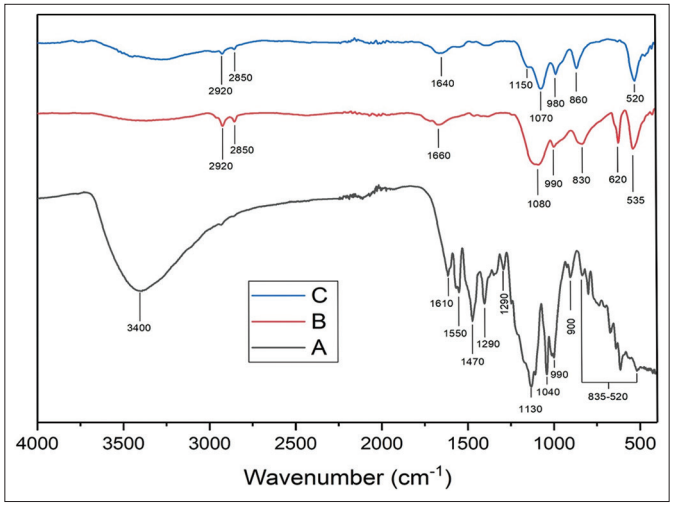

Figure 6: FTIR spectrum of (a) RB222 (Parental dye), (b) Dye metabolites at 24 h and (c) Dye metabolites at 72 h.

A validation experiment of the established design space was performed by randomly selecting parameter values from the design space ranges. Selected parameters values were 32°C, 121 ppm dye concentration, carbon source as lactose, nitrogen source as yeast extract and pH 8. The predicted mean, as determined by the software, was 91.23%. Three replicas were run, maintaining the mentioned parameters and result was found as 93.12 ± 0.3%. The result was well within the range of ± 10%, from the predicted mean. Hence, the design space was established for degradation of RB222 by our isolate *Enterobacter* Sp. CU2004.

3.9. Identification and Characterization of Dye Metabolite

FTIR spectrum of parental dye (A) was compared with dye metabolites obtained at two different time intervals 24 h (B) and 72 h (C) Figure 6. The peaks of A at functional group region of FTIR shows peaks for O-H stretching found at 3400 cm^{-1}. The range of 1600–1500 cm^{-1} shows the presence of alkene C=C stretching. Then, the peaks can be seen in the fingerprint region giving us clear indication of the functional groups. Range from 1500 to 1200 cm^{-1} shows aromatic C-C stretching, alkane C-H bending, and rocking. Range from 1200 to 1000 cm^{-1} shows alkane C-H wagging and aliphatic amine C-N stretching. Range from 1000 to 500 shows alkene C-H bending. Focusing on the spectrum of B, there are new peak formations and few peak deletions. The main peak deletion occurred at 3400 which was the O-H stretching peak. New peaks formed at 2900–2700 cm^{-1} range shows aromatic C-H stretching and alkane C-H stretching. The peak at 11640 cm^{-1} indicates the presence of alkene C=C stretching. The peak at 1100 shows the

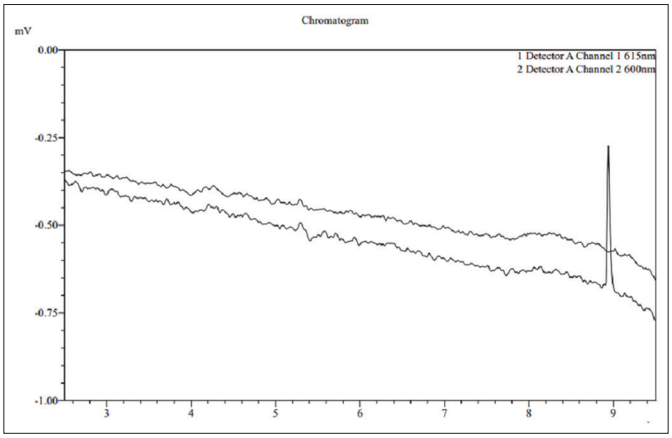

Figure 7: HPLC chromatogram showing prominent single peak for Reactive blue 222 at 615 nm.

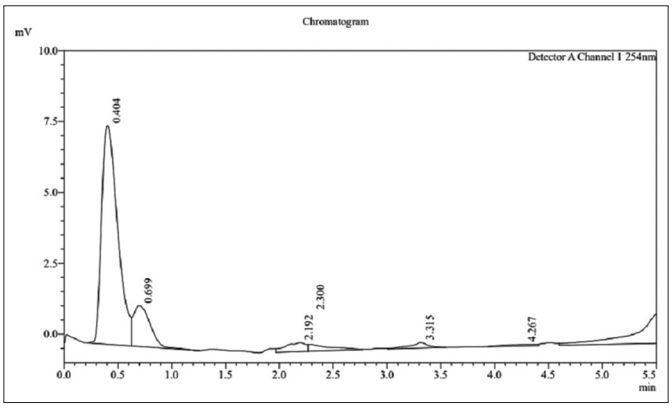

Figure 8: HPLC elute profile of reactive blue dye metabolites at 254 nm.

presence of aliphatic amine C-N stretching. The peak at 830 cm^{-1} shows the presence of ring C-C stretching and CH2 rocking. Range from 600 to 400 cm^{-1} shows the presence of ring C-C vibrations and alkane C-H bending. The spectrum of C has all the peaks similar to spectrum B except for a new peak at 1150 showing the presence of aliphatic C-N stretching and a missing peak at 620 cm^{-1}. This shows that after degradation, there is a change in the molecular chemistry of the parental dye which is evident from the FTIR comparison spectrum [48,49].

HPLC studies of the dye intermediates and the parental dye were compared with respect to their UV-Visible absorbance and retention time. Parental dye RB222 being chromogenic substance showed single peak with absorbance peak at 615 nm [Figure 7]. Microbial degraded RB222 was colorless and did not show any peak in the visible range while multiple new peaks with different retention time was observed at 254 nm [Figure 8]. This clearly states that parental dye was degraded by the isolate to different non-chromogenic metabolites [50].

GCMS analysis of dye metabolites revealed multiple peaks obtained due to fragmentation of parental dye. The compounds and its structures were determined based on (m/z) values and its biodegradation pathway was proposed [Figures 9 and 10]. High molecular weight parental dye was degraded by *Enterobacter CU2004* to lower molecular weight intermediates compounds. Initially the diazo dye undergoes asymmetric oxidative cleavage to 2-aminonapthalene-1,5-disulfonic acid(mol wt: 302.987), 3.4-diamino-5-hydroxynaphthalene-2.7-disulfonic acid (mol wt:333.993), and sodium 3 amino-5-((4-((4-((2-(sulfooxy)ethyl)sulfonyl)phenyl)amino)-1,3,5-triazin-2-yl) phenyl)amino)benzenesulphonate (mol.wt:568.012). These dye intermediates on desulfonation and N-H bond cleavage resulted

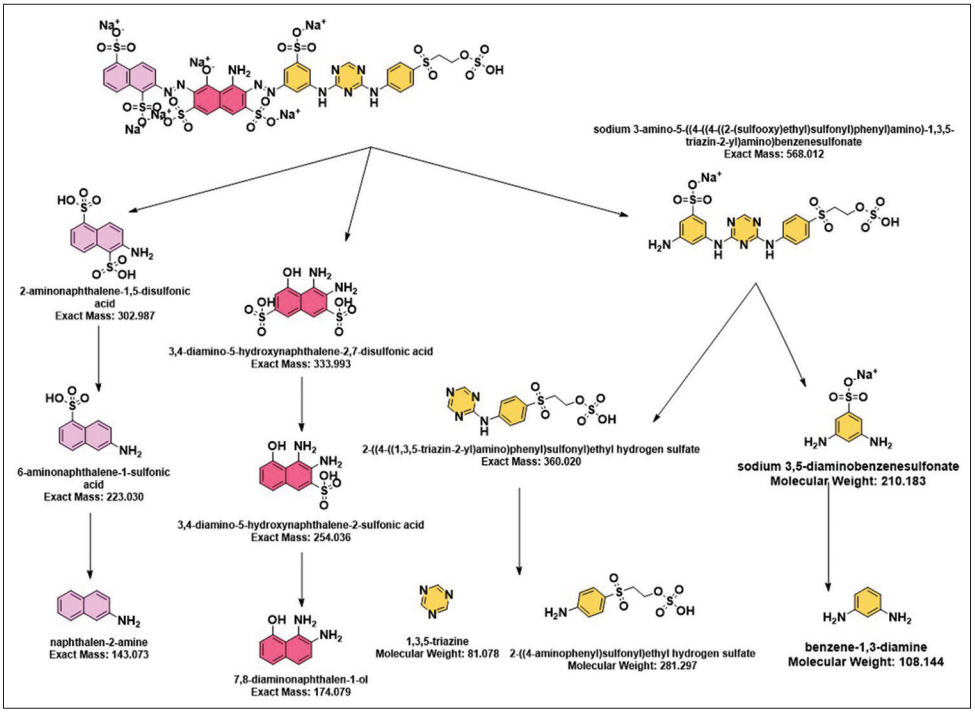

Figure 9: Proposed metabolic pathway of Reactive blue 222 by *Enterobacter* CU2004.

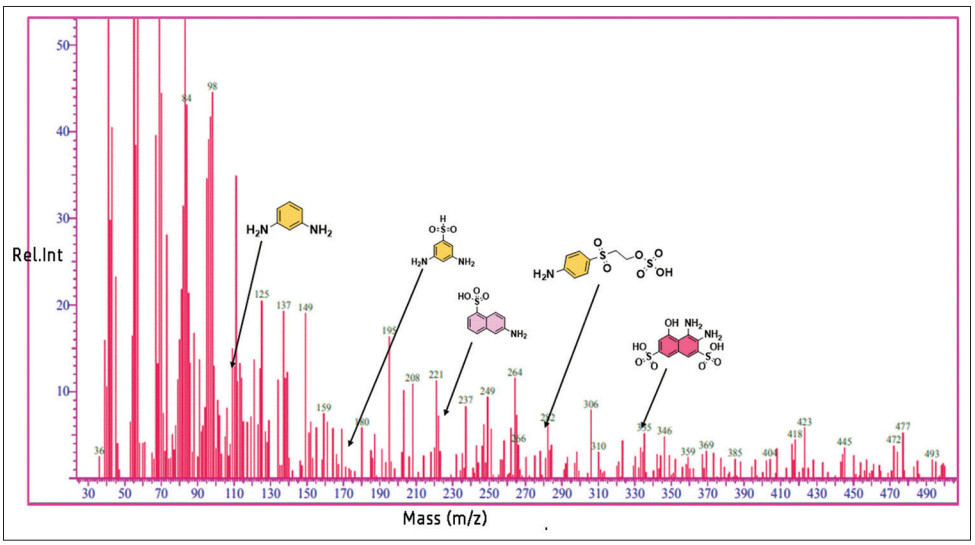

Figure 10: Gas chromatography and mass spectroscopy of degraded Reactive blue 222 by *Enterobacter* CU2004.

intermediates 6-aminonapthalene-1,5-disulfonic acid (mol wt: 302.987), 3.4-diamino-5-hydroxynaphthalene-2,7-disulfonic acid (mol wt: 333.993), and 2-((4-((1,3,5-triazin-2-yl)amino)phenyl) sulfonyl)ethyl hydrogen sulfate(mol.wt: 360.020) and sodium 3,5-diaminobenzenesulfonate (mol wt: 210.183). Fragmentation of the intermediates progress with desulfonation, N-H bond cleave and opening of rings to form 6-aminonapthalene-1-sulfonic acid(mol wt: 223.030), 3,4-diamino-5-hydroxynapthalene-2-sulfonic acid(mol wt: 254.036), 1,3,5-triazine(mol wt: 81.078), and 2-((4-aminophenyl) sulfonyl)ethyl hydrogen sulphate (mol wt: 281.297). These intermediates further fragmented to napthalen-2 amine(mol wt: 143.073), 7,8-diaminonaphthalen-1-ol(mol wt: 174.079), 1,3,5, triazine (mol wt: 81.078), 2-((4-aminophenyl)sulfonyl)ethyl hydrogen sulfate (mol wt: 281.297), and benzene-1,3-diamine(mol.wt 108.144). sequential reactions of deamination and dihydroxylation can fragment the compounds to smaller compounds like benzene, naphthalene enters gentisic acid pathway channelised to TCA cycle to completely mineralize the dye [51].

4. CONCLUSION

Degradation of most widely used complex reactive blue 222 is considered to be challenge at industrial scale; however, application of physicochemical techniques is proven to be not feasible and effective. This research identifies novel indigenous facultative microorganism *Encterobacter* Sp indigenous microbe in industrial waste. Optimizing the physical and chemical parameters by DOE approach had improved dye degradation efficiency to >90%. Our study revealed that the Bioaugmentation of *Encterobacter* Sp, CU2004 could efficiently decolorize the complex industrial dye RB222 (>90%) with the optimized parameters, namely, pH 8, 30–37°C temperature, 133–249 ppm dye concentration, lactose as carbon source, and yeast extract as nitrogen source. Successful establishment of design space further emphasizes the optimized process is scalable and therefore, can be extended to industrial scale. Considering the immense scope in the field of developing cost-effective, efficient, and scalable microbial dye degradation process, this isolate is a promising candidate in the field of Bioremediation.

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6. AUTHORS CONTRIBUTIONS

All authors contributed to the study, conception, and design. Experiments were performed by Vasantha Veerappa Lakshmaiah. Anish Nag designed the DOE part of the work. Manuscript writing and final formatting were performed by Vasantha Veerappa Lakshmaiah and Anish Nag. First draft of the manuscript was reviewed and suggestions by Sunil S more and Shobha K Jayanna conceptualized and supervised the work. Suresh had verified the data generated. All authors read and approved the final manuscript.

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8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. DATA AVAILABILITY

All the data obtained in the study are represented as table or figures.

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

12. PUBLISHER'S NOTE

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