Potential of *Aeromonas dhakensis* in bioremediation of an Azo and Untreated Dye effluent sample

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Abstract

The residual dyes in textile effluents are not efficiently remediated by conventional physical and chemical methods due to the presence of a mixture of compounds, most of which have complex structures. Consequently, the overall environmental impact of textile effluents is a global concern. Biological treatment interventions are currently encouraged since some micro-organisms can utilize dye molecules as a sole source of carbon and nitrogen. In the present study, the dye effluent collected from local industrial areas in Mumbai was screened for bacteria capable of degrading Remazol Deep Black GWF (RDB-GWF) dye. It is a reactive sulphonated diazo dye commonly used in the textile industries.

The most promising bacterial strain with dye decolorizing potential was identified as Aeromonas dhakensis based on 16S rRNA gene sequence analysis. A. dhakensis showed optimum decolorization (84.08 %) when 4% inoculum of 0.8 O.D (at 540_{nm}) was added to Luria Bertani (pH 9.0) medium containing 1% NaCl and incubated at 30°C for 24 h under shaker (120rpm) conditions. The strain showed tolerance up to 300ppm dye concentration and significantly decolorized untreated textile effluent sample. The degradation of dye was confirmed by FTIR spectroscopic analysis. Phytotoxicity studies (with Phaseolus mungo and Triticum aestivum) suggested non-toxic nature of degraded metabolites.

Keywords: *Aeromonas dhakensis*, biodegradation, decolorization, optimization, remazol deep black GWF.

Introduction

Dyes build the foundation of textile industries. They also find extensive application in paper, petroleum, food, cosmetic and pharmaceutical sectors. The unsaturated chromophores and auxochromes form the building block of dye molecules. Chromophores impart structural diversity and distinct color to dyes and auxochromes enhance binding of chromophores to fabric material and aid in uniform spreading of dyes.[4] Primarily, dyes are characterized as azo, anthraquinone, triphenylmethane and phthalocyanine types based on aromatic structure of chromophores.[24]

Among them, the azo dyes dominate the dye market by contributing to 70% of the market demand.[5] After the dyeing process, the textile effluents contain roughly 11% dyes which, along with other components, are discarded in water bodies.[43]

Due to the complex chemical structure of the synthetic dyes, they are not easily degraded and hence persist in the environment for a long time.[31] The potential hazards associated with exposure to azo dyes have raised serious global concerns and strict regulations were imposed on the disposal of textile effluents.[42] Despite various precautionary measures, the dye effluents continue to impact the environment adversely, due to the rapid growth of industrial sectors in the past few decades. In India alone, 60,000 tons of 700 types of dyes are manufactured annually and contribute to roughly 6.6% of global share only. The mean annual growth rate of dye industries in India has been between 25% - 27% since 1985.[43]

The problems associated with textile effluents include (i) presence of toxic chemicals, acids, bases, dispersants and heavy metals (ii) high BOD and COD values (iii) high costs, (iv) production of more toxic intermediates, (v) heat stability (vi) inefficient physical treatment methods and (vii) resilience to chemical treatment methods. Additionally, chemical treatments often result in production of vast amount of sludge.[18,25] For these reasons, more sustainable interventions are encouraged for environment friendly outcomes. One of the most promising strategies, in this regard, is the use of micro-organisms for bioremediation of dyes due to their potential to utilize dyes as nitrogen and carbon sources.[41] Also, due to the presence of high concentration of salts in textile effluents, salt tolerant bacterial strains have better potential in bioremediation of dyes.[3,39]

Numerous microorganisms have been reported to reduce azo dyes.[1,2,44] In addition to mineralization of dyes, microorganisms significantly reduce sludge volume.[31] Moreover, microbial strains are not dye-specific; hence, they can efficiently degrade an array of chemical structures in effluents.[3] The use of microorganisms is also cost effective. The bacterial strains are often a tool of choice for any fermentation processes due to their ease of adaptation.[29] Besides, the chances of cross contamination with bacterial strains are considerably low.

Furthermore, the genetic engineering for strain improvement has much higher success rate as compared to fungi and yeast. Although limitations like toxicity to effluents, the need for co-substrates and large scale production of microbial strains exist, the biological techniques are the most effective

strategy to address the widespread pollution due to textile effluents.[35]

The objectives of the present study were to screen for Remazol Deep Black GWF (RDB-GWF) dye decolorizing bacteria from soil samples collected from regions near textile industries and to optimize their potential. We further confirmed degradation of dye with the help of FTIR analysis and non-toxicity of degraded metabolites towards plants. A qualitative study was also carried out under optimized conditions to observe the extent of decolorization of raw (unprocessed) industrial effluent.

Material and Methods

Media and Chemicals: All chemicals and nutrient media used in the present study were of highest purity and analytical grade. The textile dye- Remazol Deep Black GWF (RDB- GWF) was obtained from the Institute of Chemical Technology (ICT), Mumbai. The other chemicals were purchased from Hi-media India Ltd., Merck India Ltd. and Difco laboratories.

Sample collection: The textile effluent samples were collected from the local dye effluent plants situated at Mahim East, Dombivali MIDC and around the vicinity of the textile industry in Kalyan, Mumbai, India.

Enrichment and isolation of dye degrading bacteria: The textile effluent samples (5 ml) were inoculated in 100 ml of sterile nutrient broth (NB) and incubated at room temperature (RT; ~30°C) under shaker (120 rpm) and static conditions for 24 h for enrichment of dye degrading bacteria. After incubation, 5 ml of enriched media was centrifuged and the pellet was suspended in 10 ml sterile phosphate buffered saline (PBS; pH 7.2) to obtain a cell suspension. The dye degraders were isolated on nutrient agar (NA) plates after suitable dilution of the cell suspension and incubated at RT for 24 h. The well isolated bacterial colonies were selected and re-isolated on NA plates to ensure purity of strains. The pure cultures were streaked on NA slants and the growth was stored at refrigerated conditions.[23]

Screening of dye degrading bacteria: To screen bacterial isolates capable of degrading RDB-GWF dye, inoculation media were prepared by adding 50 ppm concentrationof dye in NB. The pure cultures were inoculated (0.2 OD_{540nm}) in above media and incubated at RT under shaker (120 rpm) and static conditions for 24 h or further until complete decolorization was achieved. The extent of decolorization was monitored every few hours using UV-VIS spectrophotometer (Systronic 2203 model).

The assays with each strain were performed in triplicate and 5ml of aliquot was removed for monitoring. The cell free suspensions were obtained by centrifugation at 5000rpm for 20min and subjected to spectrum scan in the wavelength range of 200 and 800nm. The percentages and rates of decolorization were calculated using the formula represented below. The isolate showing maximum degradation in least time was selected as the most promising.[6,21]

$$\% \text{ Decolorization } = \frac{(A - B)}{A} \times 100$$

where A is the initial absorbance of control (un-inoculated NB containing 50ppm dye) and B is the observed absorbance of the degraded dye.

$$\text{Rate of Decolorization } = \frac{C \text{ x } (\%D)}{VX\,T} \times 100$$

where C is the initial concentration of the dye (mg), %D is the percentage of decolorization, V is the total volume of the nutrient medium used and T is the time taken for decolorization.

Identification of potential strain: The preliminary identification of all strains was done based on cultural, morphological and biochemical tests. The potential dye degrading strain was confirmed by 16s rRNA gene sequence analysis. PCR based 16S rRNA gene amplification and sequencing of the isolated bacterium were carried out using universal primers at Sai Biosystems Private Limited, Nagpur, India. The forward and reverse primers used were 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'- AAG GAG GTG ATC CAGCCG CA-3' respectively.

Optimization of factors influencing dye degradation: Besides the microbial strain, the biodegradation of any pollutant is highly influenced by the environmental factors prevalent in an ecosystem. To achieve optimum decolorization, the effects of critical environmental parameters were studied. The standard parameters used in our study were NB medium (pH 7), 30°C, shaker conditions (120rpm) and 1% inoculum adjusted to 0.1 $O.D_{540nm}$. The optimization of each of environmental parameters was done by applying one factor at a time (OFAT) approach. In this method, one variable of the system is changed at a time while keeping the others constant.[16,37,38]

Effect of different media and their composition on decolorization of RDB-GWF dye: Seven nutrient media were screened in our study to attain maximum decolorization of RDB-GWF dye by the potential isolate. These media and their composition are listed in table 1. The effects of individual components of the most suitable media were further studied with the help of deletion, addition and substitution assays. In these assays, the media components were deleted or replaced to understand their effect on the decolorization process.

Additionally, few components were added to the standard composition, in increasing concentrations, to observe increased rate of decolorization, if any. The influence of 1% carbon sources like glucose, fructose, sucrose, mannose, maltose, mannitol, arabinose, galactose, lactose, raffinose,

trehalose, xylose, ribose, inoline and sorbitol was studied on dye decolorization under previously optimized conditions.

Effect of inoculum size and density on decolorization of RDB-GWF dye: The effect of different optical densities (0.2, 0.4, 0.6, 0.8 and 1.0 at 540nm) and inoculum size (1%, 2%, 4%, 6%, 8% and 10% v/v) of potential dye decolorizing bacteria was studied in optimized media.

Effect of physicochemical parameters on decolorization of RDB-GWF dye: The varying physicochemical parameters optimized in our study were temperature (37°C, 45°C and 55°C), pH (4-11), aeration (static and 120rpm), initial dye concentration (50, 100, 150, 200, 250 and 300 ppm) and NaCl concentration (1-10%).

Confirmation of dye degradation with analytical studies: The degradation of dye molecule was qualitatively analysed using UV-Vis spectrophotometric method to observe changes in absorbance spectrum of dye after decolorization. The metabolites present in the decolorized samples were further analyzed to confirm biodegradation of dye. For this purpose, the metabolites were extracted with equal volumes of dichloromethane and concentrated by air drying. The concentrated dry residue and dye (control) was subjected to analysis by FTIR spectroscopy.

Phytotoxicity studies: The effect of intact RDB-GWF dye and its degraded metabolites were observed on seed germination. For the study, a monocot *Triticum aestivum* (Wheat) and a dicot *Phaseolus mungo* (Moong) seed were selected. They were soaked overnight in 5 ml of filter sterilized dye (dye control), degraded metabolite (test) and distilled water (negative control). After overnight incubation, the seeds were placed on sterile Whatmann filter papers and transferred to Petri plates. The seeds were regularly sprayed with tap water to prevent complete drying and monitored for germination and sprouting.[40]

Table 1
Media used for screening of dye degrading bacteria

S.N.	Media	Composition (g/l)
1	Nutrient broth (NB)[23]	Peptone (5), Beef Extract (3), NaCl (5), pH 7.2
2	M9 medium[28]	Na_2HPO_4 (0.64), KH_2PO_4 (0.6), NaCl (0.1), Ammonium chloride (0.2), pH 7.4
3	Luria Bertani (LB) broth[46]	Casein enzymatic hydrolysate (10), Yeast extract (5), NaCl (10), pH 7.5
4	Screening medium (SM)[13]	Yeast extract (10), NaCl (5), pH
5	Mineral salt medium (MSM)[38]	NaCl (1), $CaCl_2 2H_2O$ (0.1), $MgSO_4 7H_2O$ (1), Na_2HPO_4 (0.5), KH_2PO_4 (1), pH 7.2
6	Glucose yeast extract peptone (GYEP) broth[26]	Peptone (10), Yeast extract (5), Dextrose (20), pH 7.0
7	Glucose yeast extract (GYE) broth[30]	Yeast extract (5), Glucose (2), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.3), NaCl (0.01), $ZnSO_4$ (0.0016), $CoSO_4$ (0.0016), $MnSO_4$ (0.01), $CuSO_4$ (0.0016), pH 7.2

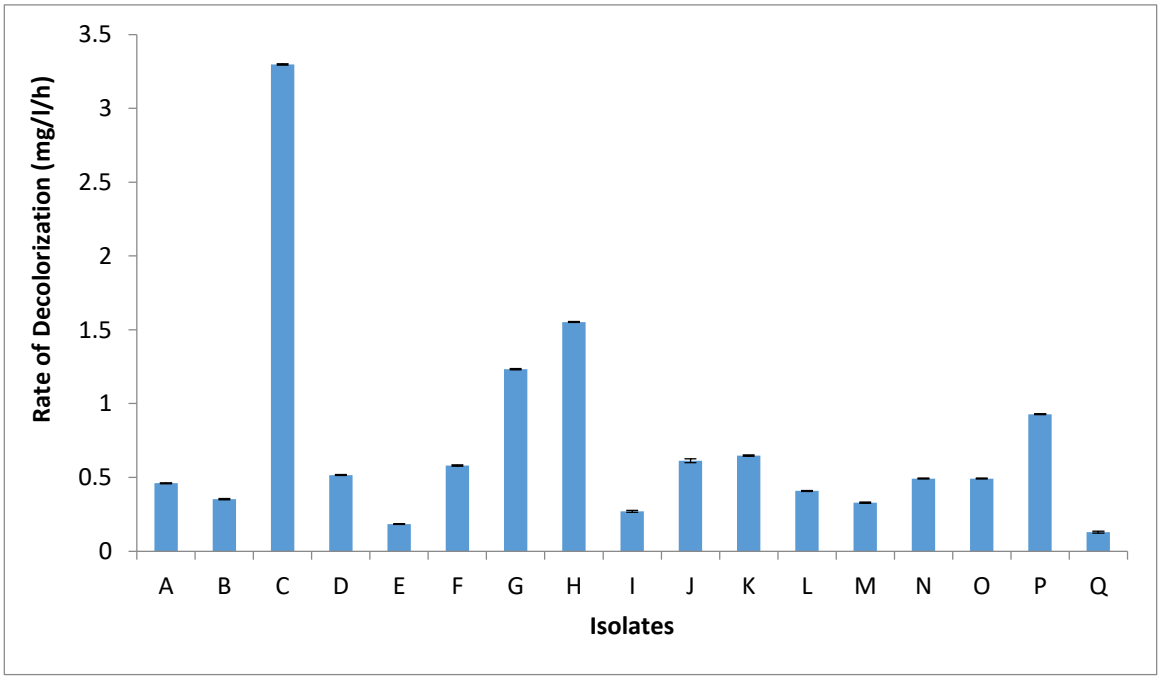

Fig. 1: Screening of potential dye degrading bacteria in Nutrient broth under standard conditions

Evaluation of decolorization efficiency of potential isolate: The ability of the potential isolate to remediate the industrial effluent (collected from Dombivali MIDC Mumbai) was evaluated under optimized conditions. The effects of optimized parameters were studied using unsterile effluent and effluent containing nutrients of optimized media.

Results

Screening and identification of dye degrading bacteria: Seventeen bacterial strains with dye decolorizing ability were isolated in our study. Fig. 1 represents the different rates of dye decolorization by all strains. Isolate C showed significantly higher rate of decolorization and hence it was selected for further studies. It was identified as *Aeromonas dhakensis* (NCBI accession number MK840994).

Optimization of media and its components: Compared to NB, better decolorization of RDB-GWF was supported in LB, SM and GYEP media by *A. dhakensis*. Maximum rate of dye decolorization was observed in LB medium (Fig. 2). A distinctly notable characteristic of *A. dhakensis* was its inability to decolorize the dye in absence of peptone. During screening of media, *A. dhakensis* showed significant dye decolorization in GYEP media. However, GYE media (where peptone is replaced by mineral solution) showed negligible decolorization. The addition assays further confirmed maximum decolorization of dye on addition of 1% peptone in LB medium.

On optimization of peptone concentration, maximum rate of decolorization was observed at 3%. However, the % increase in rate was negligible. Also, similar rate of decolorization was observed at 0.5 % and 1 % peptone concentration. Hence the lowest optimum concentration was used for further assays. Notable increase or decrease in decolorization rate was not observed during deletion and substitution assays which confirmed the stability of the media. Among the carbon sources, raffinose showed maximum decolorization. Hence, the optimized media used for further studies was LB broth containing 0.5 % peptone and 1 % raffinose.

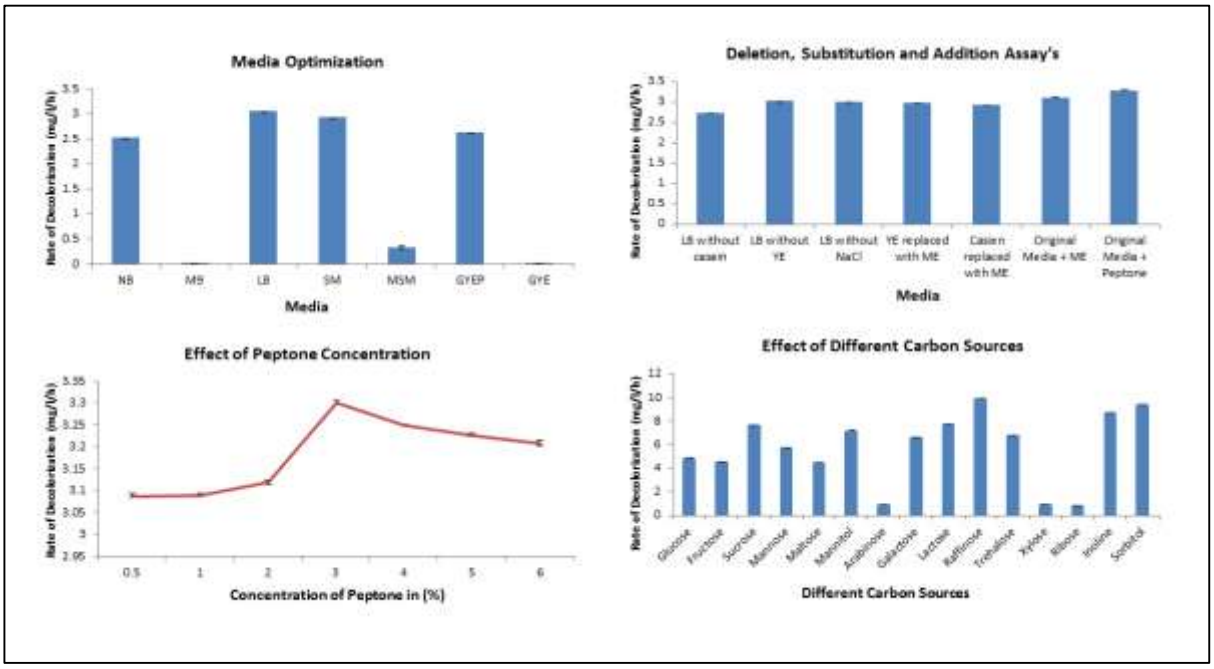

Fig. 2: Optimization of media components for degradation of RDB-GWF dye by *A. dhakensis*

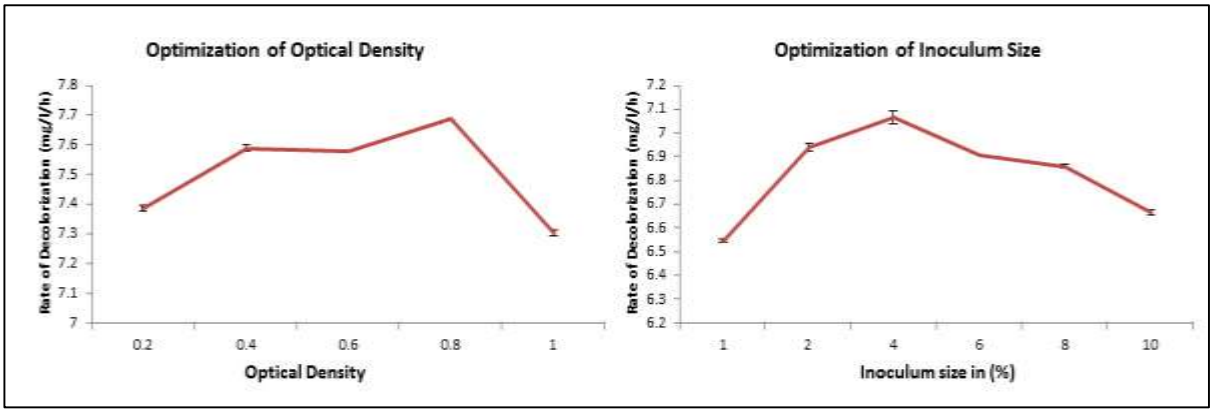

Fig. 3: Optimization of biomass for degradation of RDB-GWF dye by *A. dhakensis*

Optimization of inoculum size and density: Fig. 3 represents the effect of increasing density and inoculum size (biomass) on dye decolorization. The optimum parameter was identified as 4% inoculum size adjusted to 0.8 O.D (at 540_{nm}).

Optimization of inoculum size and density: Fig. 4 represents the effect of critical physicochemical parameters on dye decolorization. Optimum rate of decolorization was observed at 30°C, pH 9 and shaker (120rpm) conditions in 24 h. The rate of decolorization drastically reduced on further incubation and increase in dye concentration. Negligible rate of decolorization (~1 mg/l/h) was observed at 300 ppm concentration of dye indicating that this is the tolerance threshold for *A. dhakensis.*

Confirmation of dye degradation with analytical studies: The UV-Vis spectral analysis showed decrease in absorbance from 0.993 to 0.158 at 591.2 nm and appearance of new peak at 428.0 nm indicating degradation of dye. The degradation of dye was confirmed by FTIR analysis which showed difference in peaks of intact dye and degraded dye metabolite (Fig. 5).

Phytotoxicity studies: The phytotoxicity study indicated toxic effect of RDB-GWF dye since it did not support germination. Although, the degraded metabolites supported seed germination, the length of the radicle and plumule was smaller compared to controls (Table 2). Hence, the results indicated less toxicity of degraded metabolite as compared to intact dye molecule. However, further studies are required to confirm the safety of degraded metabolites.

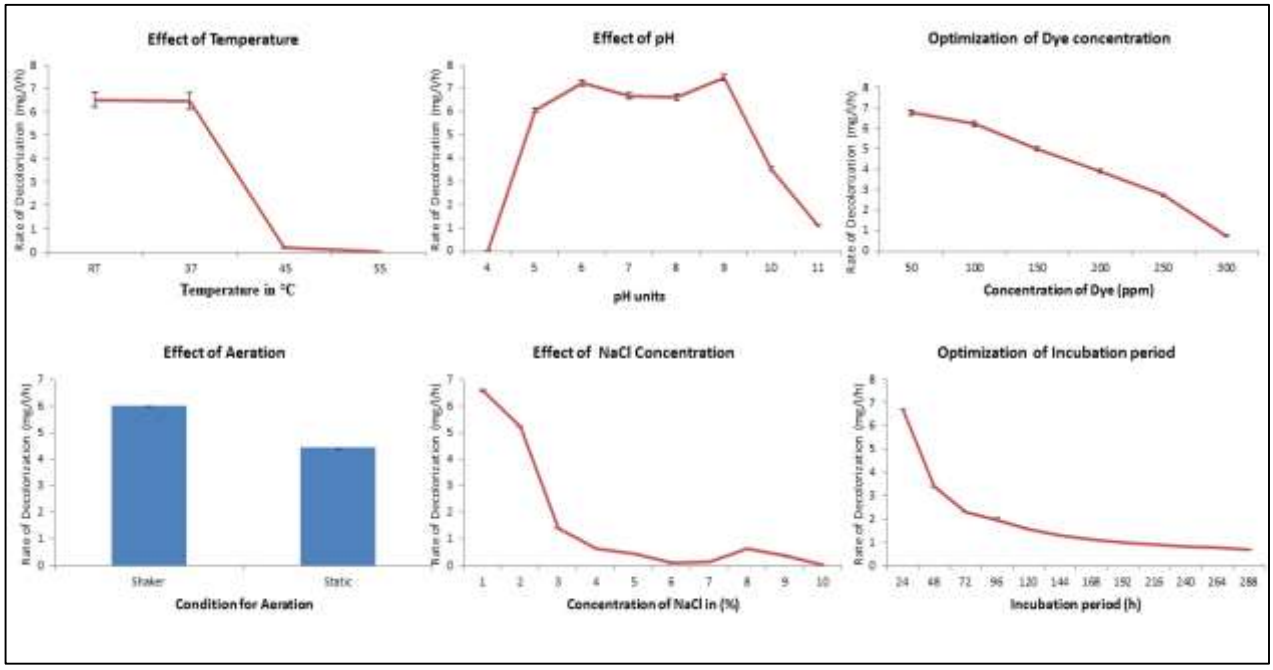

Fig. 4: Optimization of physicochemical parameters for degradation of RDB-GWF dye by *A. dhakensis*

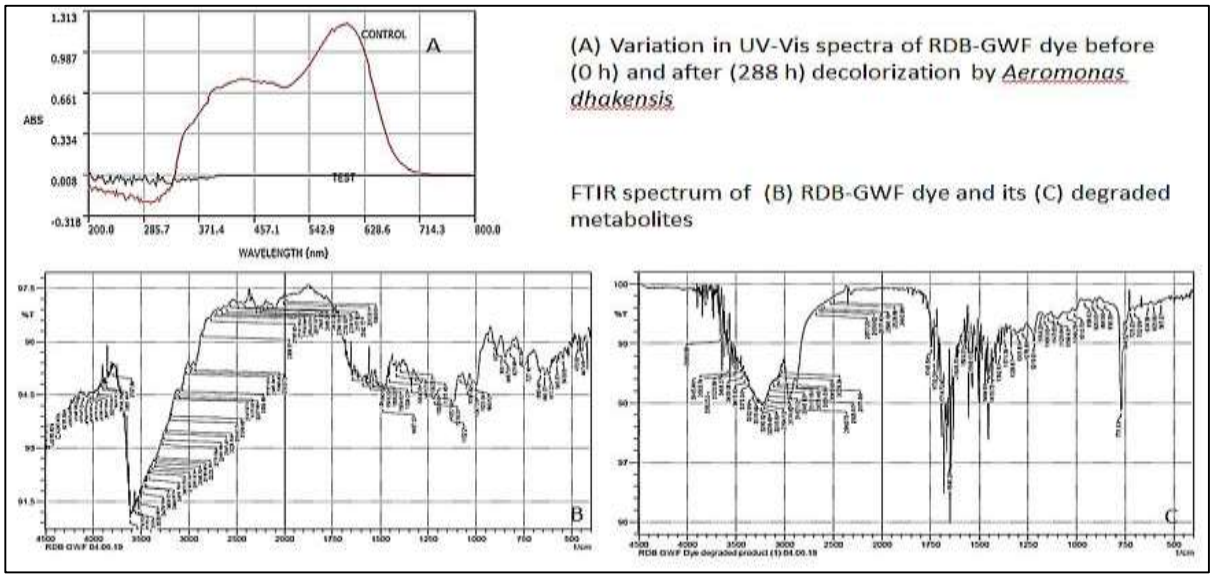

Fig. 5: Analytical studies to confirm degradation of RDB-GWF dye by *A. dhakensis*

Table 2
Phytotoxicity study of RDB-GWF dye and its degraded metabolites

Seed	Average length (cm)					
	Distilled Water		RDB-GWF dye		RDB-GWF dye metabolite	
	Radicle	Plumule	Radicle	Plumule	Radicle	Plumule
Moong	3.0	0.87	0.3	0.1	2.1	0.63
Wheat	5.0	0.76	0.34	0.1	2.0	0.41

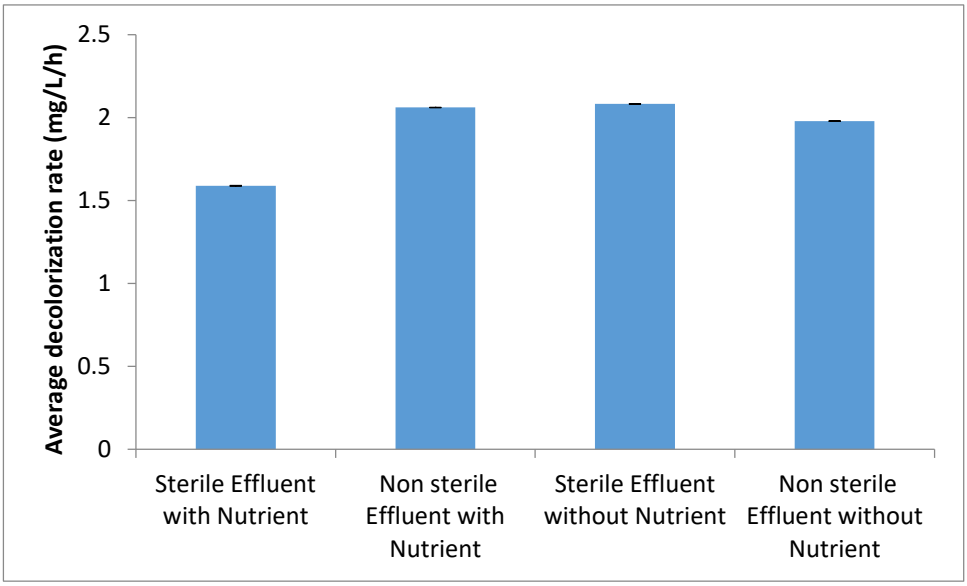

Fig. 6: Potential of *A. dhakensis* to decolorize untreated textile effluent under optimized conditions

Application of decolorization potential of *A. dhakensis* for untreated industrial effluent sample: The dye degradation ability of *A. dhakensis* was further exploited to remediate industrial effluent sample collected from Dombivali MIDC, Mumbai. Complete (100 %) decolorization was observed for sterile effluent sample with no added nutrients in 48 h, while it dropped to 76.25 % on addition of nutrients (raffinose and peptone). In non-sterile effluent sample, although complete decolorization was not observed, there was less variation between extent of decolorization observed in presence (98.90 %) and absence (94.96 %) of nutrients. The average rates of decolorization under different conditions are represented in fig. 6.

Discussion

Microorganisms are genetically programmed to adapt to changing environmental conditions and to resist the fatal effects of chemical compounds by inducing specific metabolic pathways to overcome the associated stress.[11] Hence, they are ideal tools for remediation of xenobiotics as well as organic compounds. In the present study, we aimed to screen a potential azo dye (RDB-GWF) degrading bacteria and to optimize its activity for bioremediation process. The sites near textile industries are contaminated with effluents containing dyes and other chemicals leading to natural enrichment of dye degrading microorganisms.[27]

Hence, the soil samples were collected from around industrial areas in this study. RDB-GWF dye has a complex structure comprising of N=N- bonds, substituted by a variety of groups on aromatic rings. Recent biotechnological advances have enabled enzymatic and catalytic degradation of xenobiotic compounds.[19,33,36] These processes are much more effective and faster as compared to microbial degradation processes. However, to ensure a sustainable approach, the present study was carried out with free bacterial cells rather than isolated enzymes. Besides sustainability, the use of bacterial cells offers other advantages such as system stability, natural micro-buffered environment and cost effectiveness.[10,32] Additionally, the metabolic pathways followed by different species for specific dye degradation are unique. In most cases, these pathways are not well defined and hence the enzyme sequences are unknown.[14] In such cases, bacterial cells are much better options for metabolic studies.

The 16S rRNA gene sequence analysis confirmed the isolated strain as *Aeromonas dhakensis*. It is not known to be associated with bioremediation of other xenobiotic compounds either. *A. dhakensis* is a commonly identified pathogen, mainly associated with bacteremia.[14] Characteristically, pathogenic strains show optimum growth at 37°C and pH 7. In the present study, the observations of optimum rate of decolorization coincided with the maximum biomass production of *A. dhakensis*, indicating its optimum growth at 30°C and pH 9 (Fig. 4).

Thus, there was clear evidence of a recently developed adaptation of this pathogenic strain to dye-induced stress habitat. Here, the temperature optima can be correlated with

the normal soil temperature in India (during summers) and the pH optima can be correlated with the alkaline nature of textile effluents.

Another interesting evidence for adaptation of this strain was provided by its comparatively lower tolerance to increasing dye (300 ppm) and NaCl (3 %) concentration and (low) phytotoxicity of degraded dye metabolites. Previous studies have indicated tolerance of dye degrading strains, isolated from textile industrial areas, to dye concentration between 500 to 3500 ppm and up to 10% NaCl concentration.[6, 7,8,15] Also, unlike our study that showed low toxicity of metabolites, there are evidences of formation of non-toxic metabolites on biodegradation of azo dyes.[3,9,22] It can be assumed that *A. dhakensis* strain was recently exposed to toxic dyes and has not yet developed mechanisms to completely metabolize the dye into non-toxic metabolites.

In this study, the optimum degradation parameters were identified as 4% inoculum size adjusted to 0.8 O.D (at 540_{nm}), LB médium (pH 9) containing 0.5 % peptone and 1 % raffinose and incubation at 30°C for 24 h under shaker (120 rpm) conditions. Ideally, azo dye degradation by azoreductase enzymes is reported under anaerobic or micro-aerophilic conditions during static growth. Under aerophilic conditions, it is hypothesized that the electrons generated on dye degradation are used for the reduction of O_2 instead of azo bonds by azoreductases, thus reducing the biodecolorization potential.[12,34]

Dye degradation by *A. dhakensis* under aerobic conditions indicates alternative mechanisms of dye degradation involving novel metabolic pathways and/or hydrolytic/reductive enzymes. Very few aerobic bacterial strains utilizing azo dyes under aerobic conditions have been reported. These organisms show a narrow substrate range and are more commonly associated with degradation of sulfonated aromatic amines.[47]

Considering the optimum growth media, it can be inferred that yeast extract, casein enzymatic hydrolysate and peptone in LB medium provided the necessary growth promoting nutrients which were easily assimilated by the bacterial cells and enhanced the rate of dye decolorization. In a similar study, Garg et al[20] reported optimum decolorization of reactive orange (in 72 h) in the presence of 0.7% sucrose and 0.25% peptone. Gola et al[21] and Dong et al[17] reported < 1.5% peptone concentration as significant for dye decolorization.

Conclusion

Under optimized conditions, an increase of 66.97% in rate of dye decolorization by *A. dhakensis* was observed in our study. To ensure dye degradation, the disappearance of standard peaks and appearance of new peaks were confirmed using UV-Vis spectrophotometry and FTIR spectroscopy methods. Hence, considering the potential of *A. dhakensis* in dye degradation, its efficiency was further explored for remediation of industrial effluent sample. Interestingly, the addition of nutrients in sterile effluent negatively affected the decolorization process by 24.75% suggesting their interaction with effluent components leading to decomposition of nutrients in sterile medium.

On the other hand, addition of nutrients in the unsterile effluent sample increased the decolorization rate by 3.94%. Based on these observations, it can be suggested that the indigenous microorganisms in unsterile effluent sample may have utilized the nutrients before its decomposition, and, although complete decolorization was not achieved, inoculating *A. dhakensis* to effluent treatment plants may be a practical and economic bioremediation strategy.

Overall, this study highlights the potential of *A. dhakensis* (a well characterized pathogen) in bioremediation of RDB-GWF through its unique adaptation to soil conditions contaminated with textile effluent samples. Surprisingly, among the 17 isolates, it showed best dye decolorization ability and considerable potential in bioremediation of untreated effluents. Given the adaptive response, *A. dhakensis* can prove extremely valuable in bioremediation of other xenobiotic or mixture of chemical compounds that pollute the soil environment.

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