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DEGRADATIVE DETOXIFICATION OF REACTIVE RED 195 BY BACTERIAL ISOLATES

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ABSTRACT

Synthetic dyes are widely used in textile, cosmetic, printing, drug and food processing industries. The generation and disposal of insufficiently treated coloured wastewater is environmentally hazardous. Such wastewater leads to dye contamination of the environment such as soil and natural water bodies. Biological treatment methods are cheap and offer best alternative as compared to physico-chemical methods. Bacterial decolourization and degradation is ecofriendly and cost competitive. In the present study, Reactive Red 195 was used as a model reactive azo dye. Bacteria selected for the study were identified as *Enterococcus faecalis, Georgenia sp., Bacillus cereus* and *Pseudomonas aeruginosa*. The efficiency of the decolourization of the dye by the isolates was compared with a known dye degrader *Micrococcus glutamicus NCIM 2168*. Effect of various physico chemical factors was studied to detect optima for pH, temperature and dye concentration. Degradation was confirmed by Uv-Vis absorption, TLC, HPLC and GC-MS studies. Enzymes involved in degradation studies were found to be oxidoreductases. Toxicity assay showed nontoxic nature of the degradation products. Hence the isolates having ability to degrade and detoxify the dye can be used for treatment of coloured wastewater.

Keywords: Azo dye, biodegradation, detoxification, dye industry effluent, Reactive Red 195

1. INTRODUCTION

Azo dyes consist of diazotized amine coupled to an amine or a phenol and contain one or more azo linkages. Different varieties of azo dyes are extensively used in textile, cosmetic, printing, drug and food processing industries. Azo dyes released without proper treatment represent about 15 % of the total world production, that is, 150 tons/day5 [1]. Reactive dyes, the only textile colourants designed to bond covalently with cellulosic fibers, are extensively used in textile industry because of the variety of their colour shades, high wet fastness profiles, ease of application of brilliant colours and minimal energy consumption [2]. Amongst all the dyes especially reactive azo dyes are most problematic due to their excess consumption and high water solubility. The sulphonic acid and azo group is rare amongst natural products and thus both confer xenobiotic character on this class of compounds [3]. Dyes, however, are harder to treat because of their synthetic origin and mainly complex aromatic molecular structure. Still these dyes can be removed from environment by microbial methods of decolourization and degradation which are cost effective methods.

According to Faraco et al. [4] up to 45% of the used dyestuffs are released into the process water. The wastewater may contain dyes at a concentration ranging from 10 to 200 mg/L [5, 6]. Release of dyes in the environment causes potential toxigenic, carcinogenic or mutagenic effects [7]. Hence, it was found essential to isolate bacteria having ability to degrade and detoxify the dyes.

The present work aims to isolate bacteria from dye industry effluent by enrichment culture technique. The selected isolates along with the known dye degrader were used for decolourization and degradation of the selected dye Reactive Red 195 as well as mixture of selected five dyes. Effect of various physico chemical factors was studied to detect optima for pH, temperature and dye concentration. Degradation was confirmed by UV-Vis absorption, TLC, HPLC and GC-MS studies. Enzymes involved in degradation studies were found to be oxidoreductases. Toxicity studies revealed nontoxic nature of the degradation products. These eco friendly isolates can be successfully used for treatment of dye industry effluent to reduce environmental hazards.

2. MATERIAL AND METHODS

2.1. Dye

The azo dye selected for the present study was Reactive Red 195. The dye has molecular formula $C_{31}H_{19}ClN_7Na_5O_{19}S_6$ with molecular weight 1136.31 (Fig 1). It is a sulphonated azo dye. The dye was procured from Spectrum Dyes and Chemicals Pvt Ltd, Surat, India. Analytical grade chemicals and reagents were used in the present study.

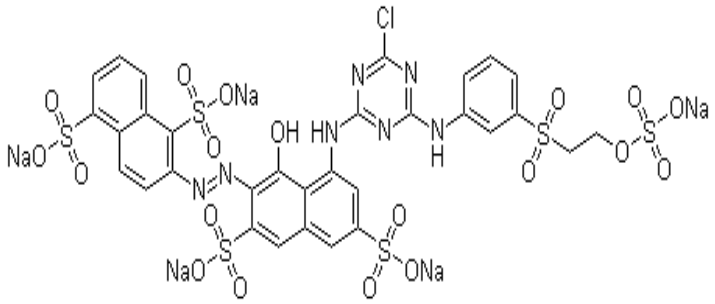

Fig. 1: Chemical structutre of Reactive Red 185

2.2. Isolation and identification of bacteria

Isolation of bacteria was done by using enrichment cultutre technique. The sources of organisms used were dye indutry effluent, dye contaminated soil, dung and sewage. Soil samples from nearby area of the dye industry, dung slurry from the digester, sewage, effluent were collected from different sites to isolate microorganisms after acclimatization to dye industry effluent. The criteria employed for the selection of sites was based on the assumption that the materials at these sites were either rich in microbial population or some had their native flora exposed to dye industry effluent. Reactive Red 195 was added as a source of carbon and nitrogen. Isolation was carried out on nutrient agar (Hi- Media M002) and the isolates were preserved on nutrient agar slants and kept at refrigerator. Genetic analysis was performed by 16s r RNA sequencing GeneOmbio Technologies Pvt. Ltd. Pune. Nucleotide sequences obatined were deposited to GenBank for accession number. Known dye degrdaer *Micrococcus glutamicus* NCIM 2168 obtained from National Centre for Industrially Important Microorganisms, NCL, Pune was used for comparing the efficiency of the dye degradation with the isolates.

2.3. Contact time and decolourization

The isolates were grown at 37°C on nutrient agar. Sterile nutrient broth was used for exploring a capability of the isolates to decolourize the selected dye Reactive Red 195. The isolates were inoculated in nutrient broth. The culture density of every isolate used was O.D.$_{600}$1. Each isolate was inoculated keeping the quantity of inoculum constant as 10% throughout the study. The dye under study was sterilized by filtration and then added to the sterilized medium. For the initial testing 50mg/L the dye was added in the medium. To study the time required for the decolourization of the dye, all flasks were incubated at 37°C up to 30 h under static condition. Three ml of the sample taken from the each media inoculated with the selected isolates, centrifuged at 6000 rpm for 20 min and used for checking the decolourization efficiency. The extent of decolourization was assessed by measuring the absorbance at λ_{max} of the dye 542 nm (Fig 2). The dye decolourization was detected by using the formula:

$$\%\text{Decolourisation} = \frac{\text{Initial absorbance - observed absorbance}}{\text{Initial absorbance}} \times 100$$

2.4. Effect of Increasing dye concentration:

To investigate the response of the isolates to the increasing dye concentration, sterile nutrient broth was inoculated with 10% inoculum of O.D$_{600}$ 1 of each isolate separately, RR 195 was added ranging from 50-500mg/L and incubated under static condition at 37°C. Experiment was carried out in triplicate. Control without culture was incubated under the same set of conditions and kept as a control tube. Percentage decolourization was calculated by using above formula (Fig 3).

2.5. Optimization of parameters for dye decolourization

In order to evaluate the optimum conditions for decolourization of the dye by the selected isolates, the medium used was sterile nutrient broth, and 10% inoculum of every isolate was used throughout the

study. Dye concentration used for optimization studies was 50mg/L. All experiments were done in the set of three. The tube containing the medium and the dye but without isolates acted as a control.

2.6. Efficiency of decolourization at different pH values

To assess the efficiency of decolourization of the isolates at different pH values, sterile nutrient broth tubes of different pH values from 3 to 8 were inoculated and incubated at 37°C under static condition. The % decolourization was calculated by using the formula and compared with the abiotic control tube of each pH values were kept (Fig 4).

2.7. Decolourization at different temperatures

Effect of temperature on decolourization was studied by using sterile nutrient broth tubes of pH 7 inoculated with the isolates and incubated at different temperatures such as 28, 37, 40, 45 and 50°C. The decolourization percentage was determined by the above mentioned formula (Fig 5).

2.8. Evaluation of decolourization in presence of different carbon and nitrogen sources

To determine the percentage of decolourization semi synthetic medium as described by Sartale et al. [8] was used. The medium is augmented with 1% of various carbon and nitrogen sources such as glucose, lactose, sucrose and starch and peptone, extracts of malt, meat and yeast as well as urea, respectively. The dye was added after filter sterilization in the medium and then inoculated with the isolates. All the tubes along with control were incubated at 37°C. The effect of these sources on decolourization was calculated by the formula [9].

2.9. Decolourization of dye industry effluent

Decolourization of dye industry effluent was carried out by sequential anaerobic aerobic method. Anaerobiaclly digested effluent was treated aerobically by the selected pure culture isolates. The extent of decolorization was measured by ADMI. The degradation of the effluent was analysed by percentage decrease in chemical oxygen and biological oxygen demand.. Uninoculated medium was used as a control.

2.10. Preparation of crude enzymes

Each isolate was grown in sterile nutrient broth at 37°C for 24 h. The cell pellets were obtained by centrifuging each culture at 10000 rpm for 20 min. The pellets were suspended in 50mM phosphate buffer of pH 7.4 and subjected to sonication. Sonication was carried out with output at 50A. Seven strokes each of 30s with 5 min interval at 4°C. This was centrifuged and the supernatant that is cell free extract was used as a source of enzymes [10].

2.11. Assay for oxidoreductses

To confirm whether the decolourization is enzymatic or not, quantitative assay of oxidizing enzymes such as laccase and lignin peroxidise, reductase such as azo reductase was carried out. The acivities were assayed spectrophotometrically in the cell free extract which is a source of enzymes. Laccase activity was detected by using 10% ABTS (2′-2′Azinobis-(3 ethylbenthiazoline-6 sulphonate) (ABTS) in acetatae buffer (0.1M) of pH 4.9 and observed for increase in optical density at 420 nm [8]. Amount of propanaldehyde forms is monitored at 300nm for detecting the amount of lignin peroxidise formed. In this 100 mM n-propanol, Tartaric acid 250mM and 10 mM hyderogen peroxide was used [11]. The assays were performed at 37°C. The tubes with all components except enzymes were used as reference blanks. All experiments were carried out in a set of three. One unit of enzyme activity was defined as a change in absorbance U/min/mg of protein of the enzyme. Protein estimation was done by biuret method. Assay of azoreductase enzyme was accomplished was using phosphate buffer 50mM of pH 7 at 50°C. The reaction was started by addition of 200µl of NADH to the mixture of 200 µl of phosphate buffer and 200 µl of the dye Reactive Red 195 and monitored at 542 nm spectrophotometrically [12].

2.12. Analysis of the degradation products

The degradation products of each isolate after decolourization of the medium were extracted twice with equal volume of dicholoromethane (DCM). The extracted residues were dissolved in HPLC grade methanol. This was used for analysis. UV –Visible spectral analysis of the extract was performed to note the changes in the absorption spectrum in the decolourized medium in the range of 400 to 800nm and compared with the absorption maximum of the dye.
Isocratic system (Shimadzu SCL 10 AVP) was used for HPLC analysis equipped with dual absorbance detector using C 18 column with methanol (HPLC grade) at a flow rate of 1.0 ml/min for 10 min at 542 nm [8].

The products of degradation of the dye were identified by GC-MS. The process was conducted with Resteck column at initial temperature of 40°C for 4 min, which was increased linearly at 10°C/min up to 270°C and held at 4 min. Injection port was having temperature of 275°C and GC–MS interface was maintained at 300°C. The helium was carrier gas; flow rate 1 mL/min and 30 min run time.

2.13. Phytotoxicity study

The study was carried out to assess the toxicity of the dye and the degradation products formed after decolourization. The phytotoxicity tests were carried out at a concentration of 400 ppm. Commonly cultivated two types of grains i.e. *Sorghum vulgare* from monocot and *Phaseolus mungo* from dicot were used in the study. The test was performed at room temperature by irrigating the 10 seeds each with sample of RR 195 and the degradation products. Tap water was used for irrigating control.. Percentage germination as well as length of plumule and radical was recorded after 7 days [8].

2.14. Statistical analysis

Statistical analysis was carried out by ANNOVA. Readings were regarded significant when P was ≤ 0.05.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of bacteria

Isolation of bacteria from original seed material (sewage, soil, sludge) acclimatized to dye industry effluent and an azo dye Reactive Red 195 were carried out by enrichment culture technique in nutrient broth with RR195 as a source of carbon and nitrogen. Decolourization was observed only in presence of carbon and nitrogen source. Out of 51 bacterial isolates, four promising isolates were selected on the basis of colour removal efficiency.

Table 1: Isolates identified and their accession number

Name of the Isolate	Accession Number
Enterococcus faecalis YZ 66	JN 129260
Georgenia Sp.CC-NMPT-T3	JN 234857
Bacillus cereus PCS 8	JN 234858
Pseudomonas aeruginosa strain MZA 85	JN 234859

The 16 s r-RNA sequencing of the selected cultures was done and the nucleotide sequences obtained were submitted to GenBank and accession numbers were obtained (Table 1). The isolates identified were *Enterococcus faecalis* YZ 66, *Georgenia* CC-NMPT-T3, *Bacillus cereus* PCS 8 and *Pseudomonas aeruginosa* strain MZA 85. The closest hit in GenBank database showed 99 to 100% similarity with the pre existing cultures.

3.2. Contact time and decolourization:

The initial pilot experiment was carried out to find out potential ability of isolates and the consortium to decolourize the dye under study. Decolourization performance was based on the structure of the dye as well as types of different biotransformation enzymes produced during decolourization. The dye molecule or its metabolite is responsible for induction or inhibition of different biotransformation enzymes that bring decolourization and subsequent detoxification process. Degradation is not only dependent on the type of the enzymes but also appropriate concentration of various enzymes [13]. RR 195, a sulphonated azo dye was 99.5% decolourized by *E.faecalis* YZ 66 within 1.5 h at 50 mg/L concentration. Wet weight of the cells during the process of decolourization was studied in order to check the ability of the isolates to grow in presence of the dyes under study. It was observed that decolourization was associated with increase in wet weight. For all isolates, decolourization was associated with a proportionate increase in wet weight indicating the ability of the isolates to grow in presence of the selected dyes. *Georgenia sp.* was able to decolourize the dye by 95.93% within 5 h. *M.glutamicus* took 18 h to decolourize 94.25% of the dye while *B.cereus* and *P.aeruginosa* decolourized 97.94% and 90.15% of the dye within 24 h, respectively (Fig 2).

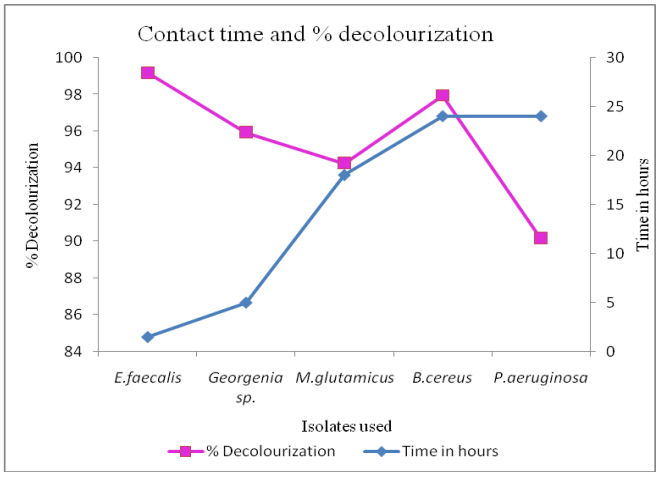

Fig. 2: Contact time and % decolourization

Ajaz et al. [14] reported 82% decolurization of Synazolred 6 HBN after 4 days incubation period. Rhodococcus strain UCC 0016 demonstrated methyl red-decolourizing activity of 100% after 24 h at static condition in comparison to Rhodococcus strain UCC 0008 which recorded 65% decolourization after 72 h [15]. Saha et al. [16] reported two *A. faecalis* species which decolorized up to 93% of Blue H/C and Red 3B dye at pH 7 and 94% of Yellow 3R at pH 8 within a period of 96 h. The isolates in the present study showed higher decolourization efficiency than the cited reports.

3.3. Effect of Increasing dye concentration

Decolourization of different concentrations of the dye ranging from 50 to 500 mg/L was studied under static anoxic condition. Among promising isolates *E.faecalis* YZ 66 was able to decolourize 96.65% of RR 195 up to 500 mg/L in 13 h. The rate of decolourization was increased up to dye concentration of 300 mg/L, above which the time required for decolourization was increased. This was observed in decolourization of the dye by all the isolates. *Georgenia* sp. decolourized 92.08% of the dye in 48 h at dye concentration 250mg/L but it took 96 h for 68.54% at 500mg/L. *B.cereus* was found to decolourize the dyes 97% in 24 h at 50mg/L. The rate of decolourization increased up to 250 mg/L but onwards it decreased. It took 72 h for about 55.42% decolourization of 500mg/L of the dyes by *B.cereus*. *M.glutamicus* showed about 90.85% decolourization in 45 h up to 300mg/L but at 500 mg/L dye concentration it required 70 h to decolourize 66.08% of the dye. *P.aeruginosa* required 76 h for 77.92% of the dye decolourization at 500 mg/L (Fig 3).

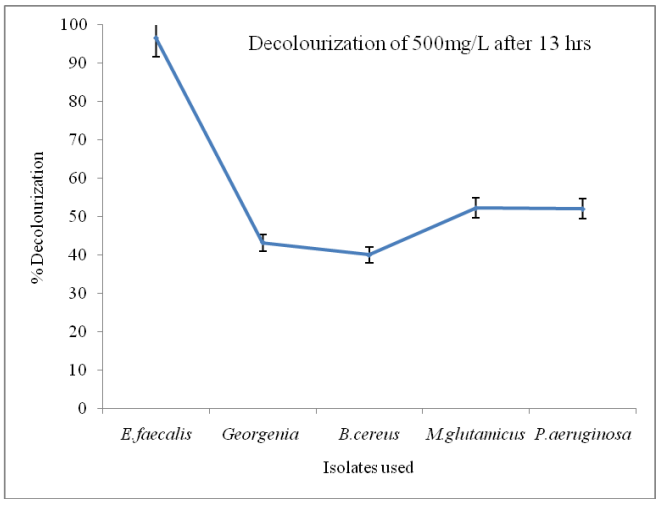

Fig. 3: Decolourization of 500mg/L after 13 hrs

This study clearly shows that, as the dye concentration is increasing, the bacterial isolates were taking longer time for decolorization. Similar studies have been reported [17, 18] in literatures where a decrease in the efficiency of decolorization was observed with increase in initial dye concentration. With subsequent increase in dye concentration toxic effect of dye and its metabolites became dominant, leading to inhibition in decolorization. The decrease in the efficiency of colour removal with increase in concentration of dye can be due to toxic effect of dye and inadequate amount of biomass to uptake this higher concentration of dye and the ability of the enzyme to recognize the substrate efficiently at the very low concentrations [19]. Decolorization significantly decreased with 500 mg /L of NSB-G and at higher concentration decolorization was severely inhibited in the study of biodegradation of reactive textile dye Novacron Super Black G by free cells of newly isolated *Alcaligenes faecalis* AZ26 and *Bacillus* spp obtained from textile effluents [20]. It was observed that in shaking conditions azo dye cleavage did not take place due to inactivity of azoreductase enzyme by the presence of oxygen in contrast static conditions made available electrons to the enzyme for the decolourization of the dye [21]. The MR decolorization increased with an increase in the dye concentration from 10 to 100 (mg/L) but decreased rapidly at the highest concentration (1000 mg/L). This might be due to the limited inherent ability of the strain to the bio-elimination process at the higher concentration [22].

3.4. Optimization of parameters for dye decolourization

3.4.1. Efficiency of decolourization at different pH values

It was observed that efficiency of decolourization depends on initial pH of the medium. Reactive Red 195(RR195) was decolorized by the isolates in varying proportions. *E. faecalis* YZ 66 deccolourized 99.36% of RR195 within 1.5 h at pH 5.0 while it showed consistent % decolourization of 96-97 from pH 6 to 8. Negligible decolourization was observed at pH 3&4. The pH 5.0 was found to be optimum for *E.faecalis* YZ 66. Significant decolourization ($P \leq 0.05$) was found at pH 5.0. *Georgenia* sp. decolourized the dye with average decolourization of 97% at pH 6-8 within 5 h. Optimum pH was found to be 7. At pH 3 and 4 it showed 11.82 and 33.97 % decolourization, respectively. *B. cereus* decolourized RR 195 maximally 97.52 % at pH 7 but the time required for decolourization was 24 h. This

isolate decolourized the dye at pH 5 to 8 with more or less equal efficiency but showed less response at pH 3 & 4. *M. glutamicus* decolourized RR 195 with average value of 97.35% at pH 5-8. Optimum decolourization was found to be at pH 5. Within 12 h it decolourized 98.59% of the dye while 40 % decolourization was shown at pH 3 & 4. The 89.45 % RR 195 was decolourized at pH 5.0 within 24 hrs by *P. aeruginosa* (Fig.4).

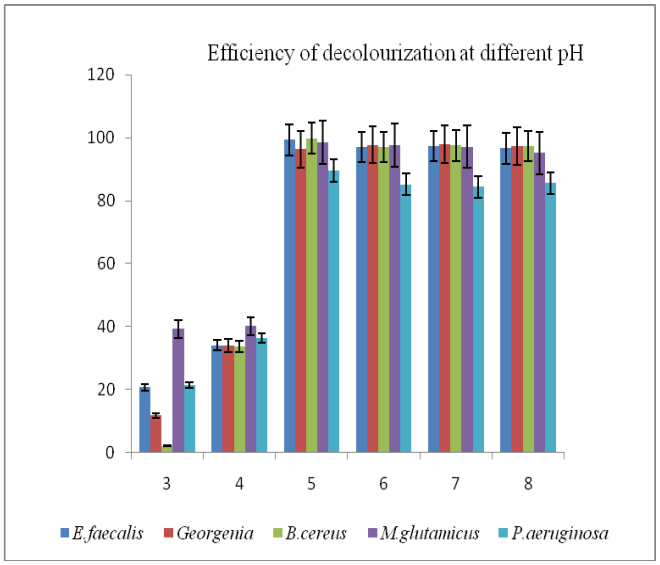

Fig. 4: Efficiency of decolurization at different pH values

Both *B badius* and *B sphaericus* demonstrated that the optimum pH for decolorization is close to pH 7 and rate of decolorization are reduced below pH 6 and above pH 8.5 [23]. The best decolourization was generally shown at pH around neutrality. The isolate showed more or less constant decolorization between pH 5 to 8. Maximum decolorization (99.00%) was at pH 6. *Bacillus spp*. ETL-2012 grows very well in the pH range of 5 to 9. Both *E. coli* and *Pseudomonas luteola* showed more or less constant decolourization from pH 7 to 9.5 [18]. Optimum decolorization activity was observed in a broad pH range of 7 to 10 by *Bacillus cereus* in biotransformation of Reactive Black 5 [24].

3.4.2. Decolourization at different temperatures

E. faecalis YZ 66 decolourized the dye under study in the range of 95 to 99 % within a temperature of 28 to 40°C. Optimum temperature for the isolate varied from 37 to 40°C. At 50 °C decolorization was found to be decreased up to 60%. *Georgenia sp.* showed 98 to 99% decolourization 37 to 40°C for the decolourization of the dye under study. *B.cereus* showed above 95 % of decolourization of dye under study at a temperature range of 37 to 40°C. Maximum decolourization of 97.51% at 37°C was shown by *M. glutamicus*. *P. aeruginosa* showed approximately similar % of decolourization of 75-85% within a temperature range of 37 to 40 °C and sharply decreased at 50 °C (Fig.5).

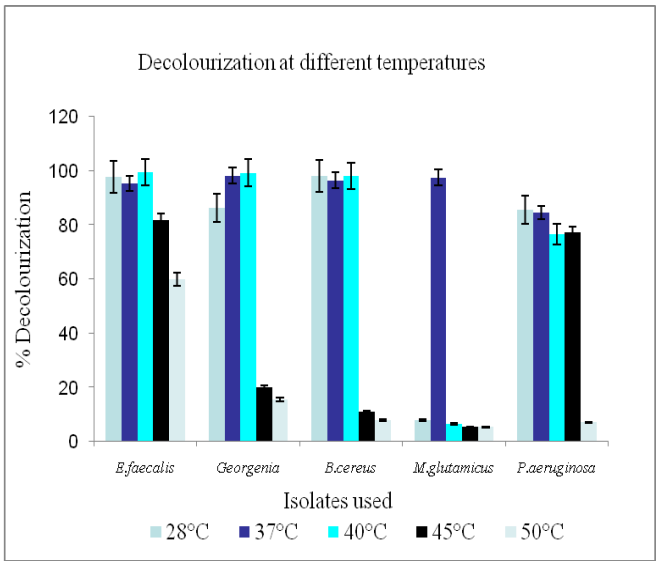

Fig. 5: Decolourization at different temperatures

Pearce et al. (2003) [25] reported that the rate of colour removal increases with increasing temperature within a defined range that depends up on the system. The temperature required to produce the maximum rate of colour removal tends to correspond with the optimum cell culture growth temperature of 35-45°C. It was reported by Mathew et al. (2004) [26] that various microorganisms showed their survival at various temperatures ranging from 25 to 50 °C. The azo reductase enzyme is relatively thermostable and can remain active up to temperature of 60 °C over a short period of time [27, 25]. The decolourization was remarkably decreased at and above 45°C. This might be due to the loss of cell viability or the deactivation of the enzymes responsible for decolorization in the study of biodecolorization of textile azo dye using *Bacillus* sp. strain CH12 [9].

3.4.3. Evaluation of decolourization in presence of different carbon and nitrogen sources

E. faecalis YZ 66 decolourized 92.5% of RR 195 within 3 h in presence of peptone and same response was shown in presence of starch within 24 h. Less

decolourization with yeast extract, malt extract, meat extract and least decolourization was observed with urea as a source of C and N within 24 h. *Georgenia* sp. decolourized 96.52% in presence of yeast extract in 5 hours and 95.13% in presence of malt extract in 24 h. In addition, it showed 69.54%, 51.21% decolourization in presence of peptone, sucrose, respectively. *B.cereus* showed 81.38% of decolourization of the dye in presence of malt extract while in presence of remaining C and N sources it showed moderate decolourization within 24 h. *M.glutamicus* showed 100 % decolourization of the dye under study in presence of sucrose followed by 90.05 in presence of glucose while 88-89% of decolourization was shown in presence of yeast extract and peptone. *P. aeruginosa* exhibited 90.61 % of decolourization in presence of glucose. Enhanced decolorization performance of Remazol Black B with Bacillus species was observed when supplied with extra carbon and nitrogen sources. Percentage decolorization (95%) was maximum with purified substrate peptone while less decolorization with other supplements of carbon and nitrogen source [18]. Higher concentration of yeast extract (8 mg/L) and lower dye concentration (25 ppm) had the highest significant effect on the percent of decolorization of Ramazol Balck 5 with *Pseudomonas* species [28]. Amongst various individual carbon and nitrogen sources, maximum decolorization was observed in MM supplemented with peptone as carbon and nitrogen source under static condition in decolorization and degradation of reactive yellow 145 textile dye by *Pseudomonas aeruginosa* and *Thiosphaera pantotropha* [29]. The results obtained in the present study were in agreement with the cited reports.

3.4.4. Decolourization of the dye industry effluent:

Amongst the selected isolates, *E. faecalis* was found to be the most efficient organism for decolourization of the dye. Hence, the same isolate was used for the study of treatment of the real dye industry effluent. The true colour of dye wastewater measured by using ADMI 3WL suggesting that *E. faecalis* could achieve higher colour removal value (72%) with moderate reduction in COD (about 40%) and BOD (about 51.94 %) after 10 days of incubation.. *Bacillus cohnii* LAP217 could exhibit significant American dyes manufactures institutes (ADMI) value reduction percentage of 79.84% during treatment of real wastewater [30].

3.5. Assay for oxidoreductases

Decolourization was based on the structure of dye as well as the different enzymes produced during decolourization. The presence of the dye either induces or inhibits the enzymes involved in decolourization. The extent of degradation was decided by the types of enzymes and concentration of the dye [13]. Since the enzyme preparations in the present study were in crude form, it highlights the combined action of studied oxidative and reductive enzymes during decolourization. Most of the enzymes showed an oxidative mechanism, which might be used in the degradation of dyes. The oxidative laccase caused an asymmetric cleavage of the dye molecule whereas azoreductase cleaved an azo bond by reductive cleavage. In the present cell free extract study though all isolates showed presence of LiP, Laccase and azoreductase in control cells. The activity of all the three enzymes was found to be enhanced during the process of decolourization.

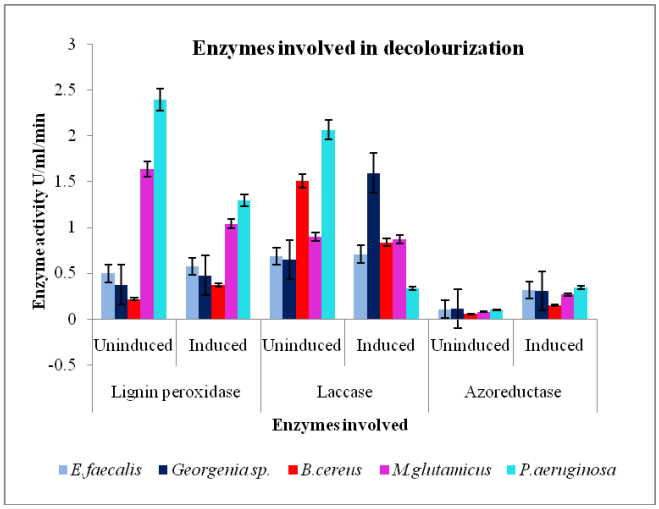

Fig. 6: Enzymes involved in decolourization

Fig. 6 showed significant increase in enzymes in induced (in presence of the dye) cells as compared to uninduced cells (control, in absence of the dye). Similar results of synergistic involvement of the enzymes in the degradation process were reported by Chen et al. [31] in biodegradation and detoxification of Direct Black G by thermophilic microflora.

3.6. Analysis of the degradation products: UV, HPLC, GCMS:

3.6.1. UV-Vis spectrophotometric analysis

UV- visible scan (400-800nm) of the culture supernatant withdrawn at different time intervals indicated the extent of the decolourization. Peaks obtained at λ_{max} disappeared at complete decolourization. The absorbance peak in the visible

region disappeared indicating complete decolourization. Evidence of the removal of dye can be observed with absorbance at λ_{max} being virtually zero after 24 h and increase in absorbance towards ultraviolet light [8, 32].

Table 2: Uv-Visible analysis of the degradation products formed by the isolates

Dye / isolate	λ in nm
RR 195	542
E.faecalis	300
Georgenia sp.	295
B.cereus	244
M.glutamicus	240
P.aeruginosa	238

Degradation product of RR 195 showed hypsochromic shift in the λ_{max}. *E.faecalis* YZ 66 showed shift from 542 nm to 300 nm while *Georgenia* showed shift to 295 nm. *Bacillus cereus* showed shift to 244 nm. *M. glutamicus* and *P.aeruginosa* showed shift to 240 and 238 nm, respectively (Table 2). Tripathi and Shrivastav , 2011 [21] reported that decolourization of dye was due to change in absorption maxima indicating dye degradation of azo dyes by bacterial strains.

3.6.2. HPLC analysis

HPLC elution profile of the dye and its degradation product/s showed peaks with different retention times. RR 195 showed two distinct and major peaks at 1.58 1.95, 2.25 and 2.96 min. Degradation products of *E.faecalis* showed two peaks at 2.56 and 2.78 min. Georgenia *sp.* showed major peaks at 2.933,3.019,3.371 min and minor peaks at 2.037 and 3.520min.Degradation product of *B.cereus* showed peak at 3.008 and 3.851 min. *M.glutamicus* showed peaks at 2.933 and 3.019 min while *P.aeruginosa* showed peaks at 2.923,3.008,3.477,3.573 and 3.797 indicating mixture of metabolites produced from the degradation of the dye (Fig. 7).

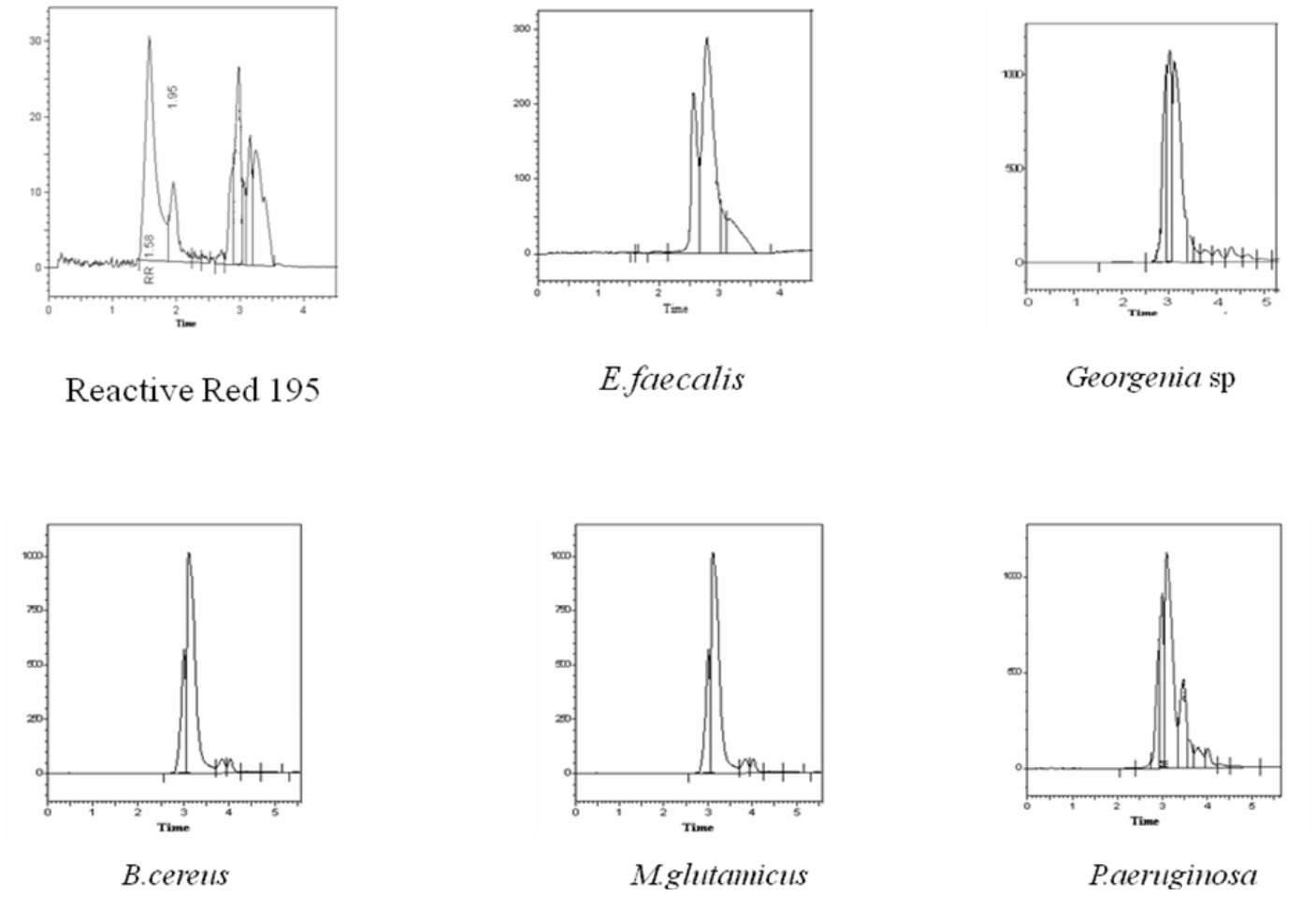

Fig. 7: HPLC analysis of Reactive Red 195 and its degraded product

3.6.3. GC-MS Analysis

GC-MS analysis of metabolites formed by an efficient degrader *E.faecalis* YZ66 was carried out to investigate the metabolites formed during the biodegradation process. Analysis of metabolites of RR 195 showed low molecular weight aromatic compound phthalic acid with molecular weight 149, desulphonated product containing aromatic ring with molecular weight 345 and trihydroxy 1naphthalene. Metabolites formed by degradation of RR 195 by the isolates were presented in Table 3. Formation of naphthalene as a final product was also reported by Dhanve et al. [33]. Thus the analytical studies confirmed the biodegradation of RR 195.

Table 3: GC-MS analysis of the degradation products

	GC-MS analysis
E.faecalis	345, 149
Georgenia sp.	154,71
B.cereus	349,154,71
M.glutamicus	349,244,217,177,154,71
P.aeruginosa	282,268,239,196,154,149,70

3.7. Phytotoxicity study:

Untreated dyeing effluent may cause the serious environmental and health hazards. In most of the cases such water was used for irrigation. This has a direct impact on fertility of soil. In case of reactive (sulphonated) dyes, biological decolourization/ degradation of dyes might form sulphonated/ unsulphonated aromatic amines, which are important groups of environmental pollutants having toxic nature as reported earlier [34, 35]. Phytotoxicity studies revealed that there was diminished germination with stunted root and shoot growth in presence of the dyes whereas in presence of metabolites, plant seeds germinated adequately as well as there was significant root and shoot development. This confirmed production of nontoxic or less toxic products of dyes under study by the isolates used in a present study.

In present study, phytotoxicity of degradation products of the selected dye by *E. faecalis* revealed significant ($P \leq 0.001$) increase in root and shoot length of *Sorghum vulgare* and *Phaseolus mungo*. It showed 90-100% germination of *Sorghum vulgare* and 80-100% germination of *Phaseolus mungo*. In presence of dye 60-80% and 50-80% germination of *Sorghum vulgare* and *Phaseolus mungo* was noted, respectively. Products formed by *Georgenia sp.*, *B.cereus*, *M.glutamicus* and *P.aeruginosa* did not inhibit germination. Root and shoot length was found to be significant ($P \leq 0.05$) confirming less toxic/ nontoxic nature of products formed (Table 4). These results supports that these promising isolates can be safely used for treatment of dye industry effluent. In the present study, these isolates have already been successfully used for aerobic treatment of anaerobically digested effluent.

Table 4: Phytotoxicity Test

Culture	Dye	*Sorghum vulgare*			*Phaselous mungo*		
		RL	SL	%G	RL	SL	% G
Control	Tap water	6.64±0.51	10.46±1.12	100	5.11±1.35	10.3±1.91	100
RR 195	RR 195	5.74±0.069	8.5±1.001	60	4.3±1.1	7.76±1.07	50
E.faecalis		8.9±1.51	11.38±0.97	90	6.3±1.74	13.07±1.22	80
Georgenia sp.		6.2±1.5	9.45±0.97	80	4.9±1.74	8.5±1.22	80
B.cereus		7.79±0.78	10.87±0.93	80	5.91±1.28	10.47±3.16	90
M.glutamicus		8.64±1.28	10.26±2.30	100	6.16±0.97	11.26±3.75	100
P.aeruginosa		7.92±0.84	10.54±1.13	90	6.0±0.88	10.73±1.11	100

4. REFERENCES

1. Gul S., Bahar G., Yildiram O. *Asian Journal of Chemistry,* 2010; **22**:3885-3894.
2. Beydilli M, Pavlostathis S. *Biodegradation,* 2005; **16**:135-146.
3. Keharia H, Madamwar D. *Ind J Exp Biol,* 2003; **4**:1068-1075.
4. Faraco V, Pezzella C, Miele A, Giardiana P, Sannia G. *Biodegradation,* 2009; **20**:209-220.
5. Pandey A, Singh P, Iyengar L. *Int Biodeterior Biodegradation*, 2007; **59**:73-84.
6. Murugesan K, Kalaichelvam P. *J. Ind J Exp Biol.,* 2003; **41**:1076-11087.
7. Legerská B, Chmelova D, Ondrejovic M. *Nova Biotechnologica et Chimica,* 2016; **15:**190-106.

8. Saratale R, Saratale GD, Chang JS, Govindwar SP. *Bioresour Technol,* 2009; **100**:3897-3907.
9. Guadiea A, Tizazua S, Meleseb M, Guoc W, Hao H, Xiad S. *Biotechnology Reports,* 2017; **15**:92-100.
10. Kalyani D, Patil PS, Jadhav JP, Govindwar SP. *Bioresour Technol,* 2008; **99**:4635-4641.
11. Telke A, Kalyani D, Dawkar V, Govindwar S, *J Hazard Mater,* 2009; **172**:298-309.
12. Maier J, Kandelbauer A, Erlacher A, Paulo AC, Gubitz G. *Appl Environ Microbiol,* 2004; **70**(2):837-844.
13. Gomare S, Govindwar SP. *J Appl Microbiol,* 2009; **106**:993-1004.
14. Ajaz M, Rehman A, Khan Z, Nisar M, Hussain S. *AMB Expr,* 2019; **9**:64-75.
15. Maniyam M, Ibrahim A, Cass A. *Environmental Technology,* 2020; **41**:71-85.
16. Saha A, Nahida S, Mohantac M, Mandald A, Haquee M. *American Scientific Research Journal for Engineering, Technology and Sciences.* 2017; **31(1):**163-175.
17. Chang JS, Chou C, Lin YC, Lin PJ, Ho JY, Hu. *Wat. Res,* 2001; **35(12):**2481-2450.
18. Shah M, Patel KA, Nair SS, Darji AM, *J Bioremed Biodeg,* 2013*;* **4**:2.
19. Das A, Mishra S, Verma VK. *J. Biochem. Technol,* 2015; **6**:962-969.
20. Hossen Z, Hussain E, Hakim A, Islam K, Uddin N, Azad A. *Heliyon,* 2019; **5**:e02068.
21. Tripathi A, Srivastava SK. *International Journal of Bioscience, Biochemistry and Bioinformatics,* 2011; **1(1):**37-40.
22. Sari I, Simarani K. *Adsorption Science & Technology,* 2019; **37(5-6):**492-508.
23. Mahbub K, Morium B, Ahmed M, Akond M, Andrews S. *J. Sci. Res,* 2015; **7:**45-53.
24. Wycliffe C, WanyonyiJohn M, OnyariPaul M, Shiundu Francis J. *Procedia,* 2019; **157**:815-882.
25. Pearce C, Lloyd JR, Guthrie JT. *Dyes Pigm,* 2003; **58**:179-196.
26. Mathew S, Madamwar D. *Appl Biochem Biotechnol.* 2004; **118**:371-381.
27. Jadhav J, Kalyani DC, Telke AA, Phugare SS, Govindwar SP. *Bioresour Technol,* 2010; **101**:165-173.
28. Hashem R, Samir R, Essam T, Ali A, Amin M, *AMB Expr.* 2018; **8**:83 13568-018-0616-1.
29. Garg N, Garg A, Mukherji S. *Journal of Environmental Management*, 2020; 263 a.
30. Mishra S, Mailti A. *Bioremediation Journal,* 2020**; 24(1):**1-20.
31. Chen Y , Feng L , Li H , Wang Y, Chen G , Zhang Q. *Bioresour Technol,* 2018*;* **250**:650-657.
32. Dawkar V, Jadhav UU, Jadhav SU, Govindwar SP. *J Appl Microbiol*, 2008; **105**:14-24.
33. Dhanve R, Kalyani DC, Phugare SS, Jadhav JP, *Biodegradation,* 2009; **20**:245-255.
34. Tan N, Van LA, Van EM, Slenders P, et al. *Biodegradation,* 2009; **16**:527-537.
35. Gottileb A, Shaw C, Smith A, Wheatley A, Forsythe S. *J Biotecnol,* 2003; **101**:49-56.