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Biodegradation of Reactive Red 195 By A Novel Strain *Enterococcus casseliflavus* RDB_4 Isolated from Textile Effluent

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ABSTRACT

Reactive Red 195 (RR-195) is a sulphonated azo-dye used extensively in textile industries. The current study was carried out to investigate the decolourisation of RR-195 dye using a novel bacterial strain, in an attempt to overcome the environmental hazards caused due to the unopposed industrial laws and substandard waste management systems. The most promising bacterial strain isolated from textile effluent was identified as *Enterococcus casseliflavus* RDB_4 by 16s rRNA analysis. It showed complete decolourisation of 50 ppm dye in optimized M9 medium (pH 7) supplemented with 3% yeast extract, within 4 h at 35°C using inoculum size of 0.2 $O.D_{530nm}$ under static conditions. Moreover, it showed tolerance to high NaCl (1 to 6%) and dye (1000 ppm) concentrations in batch as well as continuous culture systems; thus making it ideal for industrial dye waste management. Furthermore, the biodegradation of RR-195 dye was confirmed by high-performance liquid chromatography and Fourier transform infrared spectroscopy analysis into nontoxic metabolites ensured by ecotoxicity studies.

INTRODUCTION

An immense obsession with exquisite lifestyle has pressurized the textile industry to use coloured dyes in order to chaperone the appearance of finished goods. A similar situation is prevalent in other industries producing paper, leather, food, drug, cosmetics, fashion products and plastic merchandise (Thakur 2006). These profit-driven industries have collectively managed to crown the dye-related trades as the most important sector of the chemical industry and contaminate the environment in the process.

Dyes are basically derived from petroleum products containing unsaturated chromophore group of molecules which are capable of absorbing light in the visible region of the electromagnetic spectrum (400 to 750 nm). Most dyes are recalcitrant molecules that chemically lock the colour onto fibres or other materials and resist decolourisation on exposure to soap, water, light or other mild chemical agents (Robinson et al. 2001, Banat et al. 1996, Plumb et al. 2001). It is estimated that approximately 7×10^5 metric tons of 10,000 different dyes are produced annually, worldwide (Thakur 2006). This roughly quantifies to more than 1 million tons of dye production; of which about 40% ends up in wastewaters. Among these, azo-dyes constitute nearly over 70% of the synthetically produced dyes (Suteu et al. 2010).

The extensive utilization of azo-dyes by industries can be attributed to the complex structure of the dye that allows the possibility of diverse shades without compromising its stability (Neil et al. 2000). Azo dyes generally contain at least one N=N double bond and are connected to benzene and naphthalene rings. In certain cases, a sulphonate substituent group may be present, in which case it is termed as a "sulphonated azo-dye". These complex structures may be favourable for industrial requirement; however, it poses a serious challenge for waste disposal systems.

The presence of dyes in textile effluents significantly affects the photosynthesis activity in aquatic life because of reduced light penetration. The luminosity can be affected even by the presence of less than 1 ppm dye concentration in water bodies. Along with other contaminants, these dyes also aid in subsequent depletion of dissolved oxygen concentration and hence alter the aquatic ecosystem (Moreira et al. 2004). The resilience to disintegration observed in the complex chemical structure of these dyes further compromises the environmental safety. Hence, regulatory acts affirm the prerequisite treatment of industrial effluent containing synthetic dyes to its discharge in the environment.

The routinely operated physical and chemical techniques of effluent treatment including sedimentation, filtration, segregation, coagulation/flocculation, etc., have been in practice for over decades. However, they suffer several drawbacks associated with the excess use of chemicals and high expenditure. Besides, these techniques are labour intensive and trigger problems with sludge disposal. Also, many times,

the disappearance of colour is believed to be an indication of dye degradation. This can further aggravate the intensity of the situation, since the incomplete degradation of several dyes may produce end products that are more toxic than the native dyes. The intermediate compounds produced during the breakdown of complex dyes are aromatic amines with constituent side groups. Contamination in such cases may not be perceptible, but it creates a larger and more deleterious problem (Brown & Stephen 1993). Thus, the complete degradation of dyes from aromatic amines to simple compounds is a priority research venture that satisfies the current need of the environment. Most evidently, the biological processes of effluent treatment may be able to surpass these drawbacks and provide a more economical and environment-friendly alternative to the existing technologies (Jacob & Azariah 2000, Ngwasiri et al. 2011). There have been reports of bacterial, fungal, yeast and algal (Kurade et al. 2017, Pillai 2017, Chen & Ting 2017, Dellamatrice et al. 2017) degradation of dyes into simple compounds. Although the biological approach of effluent treatment may face challenges like alkaline pH and the presence of high amounts of inorganic salts, it can be prevented by exploiting the salt-tolerant and alkaliphilic microorganisms.

The present study focuses on the use of a novel salt-tolerant and alkaliphilic bacterium *Enterococcus casseliflavus* RDB_4 isolated from textile effluent, to degrade one of the frequently used textile dyes i.e., Reactive Red 195 (RR-195). It is a sulphated azo-dye containing reactive groups which are often a heterocyclic aromatic ring substituted with chloride. It is mainly used for dyeing of cellulose fabrics, but other uses also include viscose fibre printing. Considering the possibility of toxic nature of biodegraded components of RR-195, our study also emphasizes the toxicological investigation of pure and decolourised dye using germinating seeds of *Phaseolus mungo* and *Triticum aestivum*.

MATERIALS AND METHODS

Chemicals and media used in the study: The dyes used in our study viz., RR-195, Disperse blue B3, Reactive brilliant blue 198, Reactive brilliant blue 19, Reactive Red 120, Direct red 5B, Reactive Orange 16, Reactive black 5, Coracion green HE4B, Blue MEBRF 150%, Navy blue ME2G, Navy blue MEBL, Reactive yellow, Coracion red HE3B-N, Coracion orange HE2R, Orange ME2R and Corafix red ME4B were procured from Atul dyes, Mumbai, India. Other common dyes used in the laboratory such as methylene blue and congo red were procured from Merck chemicals. All other growth media like Nutrient Broth (NB) and media components were obtained from Hi-media Pvt. Ltd., Mumbai, India. Dye stock solution of 10 mg/mL was prepared in sterile distilled water and used for further experiments.

Sample collection: The screening of dye decolourising and degrading bacteria was carried out from the textile effluent collected from Dhanlakshmi Textile Mill, Mumbai.

Enrichment of dye degraders: A 10 mL volume of the textile effluent was diluted 1:10 using sterile phosphate buffered saline (pH 7.2). The solid particles in the above mixture were allowed to settle for a period of 1 h and 10 mL of the supernatant was inoculated into two flasks, each containing 90 mL of NB incorporated with 50 ppm of RR-195 dye. For enrichment of dye degraders, one of the inoculated media was kept on a rotary shaker (150 rpm) while the other was kept under static conditions and incubated at 30°C for 7 days.

Isolation of dye degrading bacteria: Isolation of bacterial species was carried out from the flask showing decolourisation on Luria Bertani (LBM) agar medium containing 50 ppm RR-195 dye. The promising isolate showing most efficient decolourisation in the shortest time period was selected for the present study and maintained on LBM agar slants containing 50 ppm RR-195 dye.

Identification of dye degrading bacteria: Primary identification of the isolate was done on the basis of morphological, cultural and biochemical tests. The strain was confirmed by 16s rRNA gene sequence analysis. PCR based 16S rRNA gene amplification and sequencing of the isolated bacterium was carried out using universal primers at SciGenom Labs Pvt. Ltd., Kerala, India.

Dye decolourisation assay: For decolourisation studies, 20 mL sterile LBM broth containing 50 ppm RR-195 dye was inoculated with 1 mL of 18-24 h old test isolates (0.2 $O.D_{530nm}$) and incubated at 30°C under static and shaker conditions until decolourisation was observed. Following incubation, the cell-free supernatant was collected by centrifuging small aliquots (3 mL) of the inoculated media at 10,000 rpm for 10 min. The decolourisation activity was expressed in terms of percentage (%) decolourisation using a UV-Vis spectrophotometer and determined by monitoring the decrease in absorbance at the maximum wavelength of dye (540 nm). The uninoculated culture medium containing 50 ppm RR-195 dye was used as the experimental control to check abiotic loss of the dye, and sterile LBM broth without test culture or dye was set up as blank.

All experiments were carried out in triplicate and the mean values were represented. The data obtained were checked statistically for the standard error and standard deviation. The % decolourisation was calculated using the following equation:

$$\% \text{ Decolourisation} = \frac{A - B}{A} \times 100$$

Where, A is the initial absorbance of control dye and B is the observed absorbance of the decolourised dye (Dhanve et al. 2008).

Optimization of various physico-chemical parameters: The biological decolourisation and degradation of any pollutant are affected by several environmental factors like pH, temperature, oxygen, incubation time, substrate concentration, etc. In our study, the optimization experiments were initiated by determining the optimum nutrient medium for dye decolourisation assay. The different nutrient media used in our study were LBM, mineral salt medium, NM9 medium, M9 medium, synthetic medium and NB. The decolourisation was monitored by using 20 mL of various culture media containing 50 ppm of RR-195 dye, inoculated with 1 mL of 18-24 h old test culture (0.2 $O.D_{530nm}$) and incubated at 30°C under static and shaker conditions until decolourisation was observed.

The optimization of physico-chemical parameters for biodegradation of RR-195 was investigated by varying one parameter at a time while keeping the others constant. These varying parameters included the initial optical density of test isolate ($O.D_{530nm}$ 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0), temperature (5°C, 30°C, 35°C, 45°C and 55°C), pH (1 to 10), concentration of NaCl (1 to 10%), aeration i.e., static or shaking (150 rpm) and initial dye concentration (50 ppm to 1000 ppm). We also carried out an addition, substitution and deletion assay of organic nitrogen sources to check the effect of an individual component of LBM broth showing most effective dye decolourisation. For this purpose, in one set of experiments, the yeast extract and tryptone in the LBM broth was substituted with meat extract (0.5%) and peptone (0.5%) respectively and in the other set, the same was supplemented to LBM broth. To check the effect of an individual component of LBM broth on the dye degradation, the individual component of the medium was deleted one at a time and the decolourisation assay was carried out.

In addition, the M9 medium was also used to study the effect of varying concentrations of yeast extract (1-5%), electron donors (sodium lactate, sodium pyruvate, sodium formate, sodium acetate and various carbohydrates viz., glucose, lactose, maltose, mannitol, and sucrose), and electron acceptors (ammonium nitrate, sodium nitrate, sodium nitrite and potassium nitrate) to examine their effect on dye decolourisation.

Moreover, the effect of pre-grown cell mass was also studied on decolourisation of RR-195 dye under optimized conditions. The results for optimization assays were recorded in the shortest time during which maximum decolourisation of RR-195 was observed. At the end of incubation period, cell-free supernatant was collected and % decolourisation was determined to optimize the culture conditions.

Mode of decolourisation: In order to determine the possibility of decolourisation due to extracellular adsorption by microbial cells over biodegradation, the % decolourisation of RR-195 dye by heat-killed and live test culture were assessed in our study. For this purpose, the test culture was inoculated into sterile M9 medium containing 3% yeast extract and allowed to grow at 30°C for 24h. Following incubation, the M9 medium was divided into two equal portions; one of which was autoclaved to kill the grown test culture. Later, 50 ppm of RR-195 dye was added to both the portions and incubated at 30°C until decolourisation was observed (Shah & Patel 2014).

Repeated dye decolourisation in the fed-batch process with pregrown cell mass: In order to study the continuous decolourisation potential of the test culture, a repetitive fed-batch system was set up. In this system, a pregrown cell mass of the test culture was inoculated in optimized culture media and incubated at 35°C. A 50 ppm RR-195 dye concentration was added repeatedly to this system after an appropriate time interval when complete decolourisation was observed (Sahasrabudhe & Pathade 2012). This cycle was repeated until significant decolourisation activity was observed.

Immobilization of test culture in calcium alginate beads: In order to carry out immobilization, 1 mL of the test isolate (5% w/v) was added to 100 mL of sterilized and chilled sodium alginate solution (4% w/v). This alginate-cell mixture was extruded drop by drop, using a 10 mL pipette, into a chilled and sterile 0.2 M $CaCl_2$ solution. Gel beads of approximately 2 mm diameter were obtained which were hardened by re-suspending it into a fresh $CaCl_2$ solution for 2 h with gentle agitation. Finally, these beads were washed with sterile distilled water and used for decolourisation experiment under optimized conditions (Cheetham & Bucket 1984, Usha et al. 2010).

Ecotoxicity studies: In order to assess the toxicity of the RR-195 dye and its degradation products, their effect was studied on germination of *Phaseolus mungo* and *Triticum aestivum* seeds. The investigation was carried out by exposing 10 seeds each to the 5 mL solution of pure dye (600 ppm) and its degradation products respectively.

The degradation products of RR-195 dye were extracted with equal volume of dichloromethane, dried and dissolved in distilled water. The length of plumule (shoot) and radicle (root) was recorded after seven days and compared with a control set which was soaked in distilled water (Dhanve et

al. 2008, Usha et al. 2010. Patil et al. 2010).

Analytical methods: Metabolites produced during biodegradation of the RR-195 dye were extracted with equal volumes of dichloromethane and concentrated using rotary vacuum evaporator (Celik et al. 2011). The concentrated dry residue of the extracted metabolites and dye (control) were analysed by a high-performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR). The HPLC analysis was performed at IIT (Mumbai), and FTIR analysis was carried out in our laboratory using Agilent Technologies Cary 630 instrument. The Agilent Resolution Pro software was used for the spectral scan.

Decolourisation spectrum of various textile dyes and dye mixture by the test isolate: Textile effluent is a mixture of various dyes; hence it is necessary to exploit the ability of test isolates to decolourise diverse dyes and even mixtures of dyes. For this purpose, the % decolourisation of the different dyes (50 ppm) such as Dispersive blue B3, Reactive brilliant blue 198, Reactive brilliant blue 19, Reactive Red 120, Direct Red 5B, Reactive Orange 16, Reactive black 5, Coracion green HE4B, Blue MEBRF 150%, Navy blue ME2G, Navy blue MEBL, Reactive yellow, Coracion red HE3B-N, Coracion orange HE2R, Orange ME2R, Corafix red ME4B was determined in optimized culture medium over 5 h. Various combinations of different dyes (50 ppm of each dye) were also used in our study.

RESULTS AND DISCUSSION

RR-195 decolourisation studies: Seven distinct isolates were obtained from the dye effluent sample and all these were subjected to decolourisation assay. Fig. 1 represents the decolourisation activities of test isolates. Isolate 3 showed the most promising decolourisation activity under static conditions, and hence was selected for the present study.

Identification of the isolate: The cultural, morphological and biochemical tests identified the promising isolate (isolate 3) as *Enterococcus casseliflavus* and 16s rRNA analysis also confirmed the same. The nucleotide sequence analysis of the isolate was done at BlastN site on NCBI server (http://www.ncbi.nlm.nih.gov/ BLAST) and corresponding sequences were downloaded. The identified isolate was deposited as *Enterococcus casseliflavus* RDB_4 at National Centre for Biotechnology Information (NCBI) with an accession number KP268598.

Optimization of physico-chemical parameters for dye decolourisation: The results for optimization of physico-chemical parameters for dye degradation are represented in Figs. 2-14. The optimized culture condition for the growth of *E. casseliflavus* RDB_4 (2.0 $O.D_{530nm}$) to effectively decolourise 50 ppm of RR-195 dye was achieved when it was incubated at 35°C for 10 h under static conditions using LBM (pH 7). However, the most effective decolourisation was observed in M9 medium containing 3% yeast extract that resulted in complete decolourisation of 50 ppm RR-195 dye within 4 h.

Prior to optimization of physico-chemical parameters, it is essential to optimize the nutrient media in order to ensure maximum growth, and hence degradation of the dye. In our study, the decolourisation potential of *E. casseliflavus* RDB_4 was studied in various liquid media indicated in Fig. 2. Among the tested media, LBM supported maximum (88.24%) decolourisation of RR-195 dye in 10 h. The synthetic and mineral media, on the other hand, supported negligible decolourisation of RR-195 dye. Our previous study also showed effective biodegradation of Reactive blue 172 by *Shewanella haliotis* DW01 in LBM (Birmole et al. 2014). Other authors have also indicated the positive effect of tryptone and yeast extract in LBM that acts as readily available carbon as well as nitrogen sources for bacterial growth and hence promote effective decolourisation of the dye (Dhanve et al. 2008).

One of the important parameters for any microbiological process is the initial density of microbes used for the experiment. In cases where a lower density of starter culture is used, their potential activity may not be exploited and in cases where more than optimum concentrations are used, the nutrients may become exhausted before effective activity initiates. In our study, a maximum decolourisation of 83% was obtained with 2.0 $O.D_{530nm}$ (Fig. 3) in 10 h. However, a culture density of 0.2 was used in further experiments, since there was no significant change observed in the decolourisation process with very dense cultures. Also, using a lower cell number is economical and feasible for the degradation process.

The optimum growth temperatures enable proper microbial metabolism and hence release an optimum concentration of enzymes required for the degradation process. In our study, dye decolourisation process was inhibited at low (5°C) and high (55°C) temperatures probably due to inactivation of enzymes or loss of cell viability. Our study showed significant decolourisation of RR-195 dye by *E. casseliflavus* RDB_4 at temperatures in the range of 30 to 45°C (Fig. 4) in 10 h with maximum decolourisation (97.99%) observed at 35°C. This also confirms that the degradation of dye is due to microbial metabolism that is dependent on temperature, and not due to adsorption (Bhatt et al. 2005). These results are in accordance with those of Sahasrabudhe & Pathade (2012), who reported maximum decolourisation of azo dye at 40°C by *Enterococcus faecalis* YZ66. Other studies have

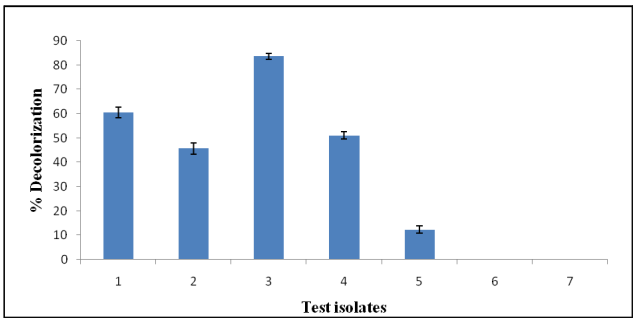

Fig. 1: Decolourisation of RR-195 dye by test isolates.

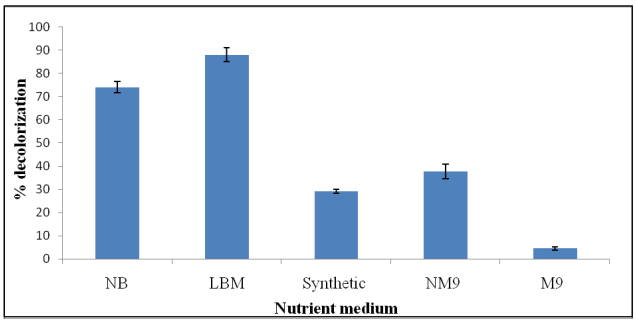

Fig. 2: Optimization of nutrient media for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

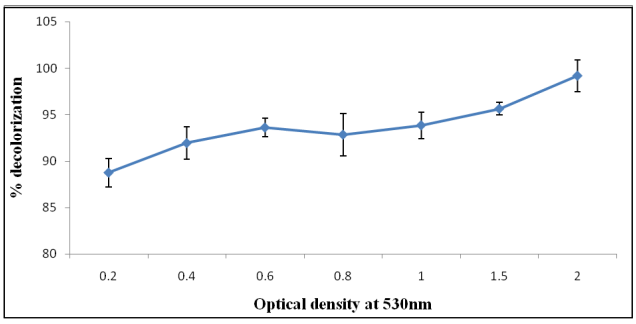

Fig. 3: Optimization of optical density for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

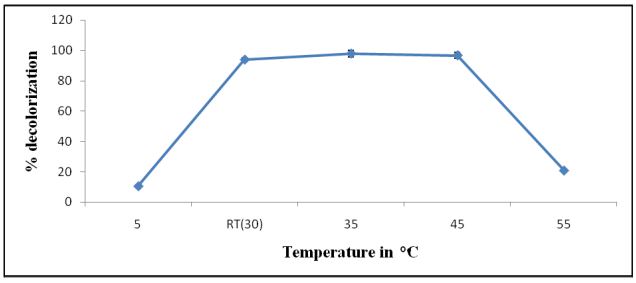

Fig. 4: Optimization of temperature for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

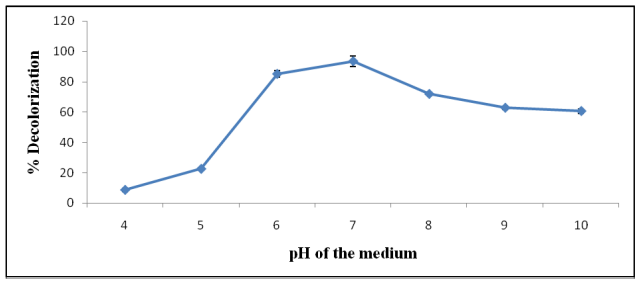

Fig. 5: Optimization of pH for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

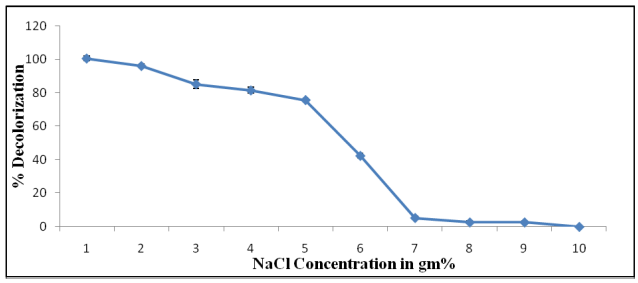

Fig. 6: Optimization of NaCl concentration for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

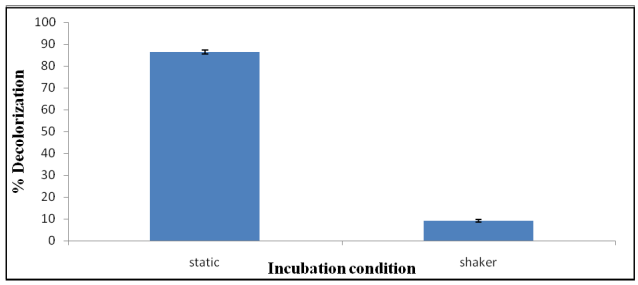

Fig. 7: Optimization of incubation conditions (aeration) for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

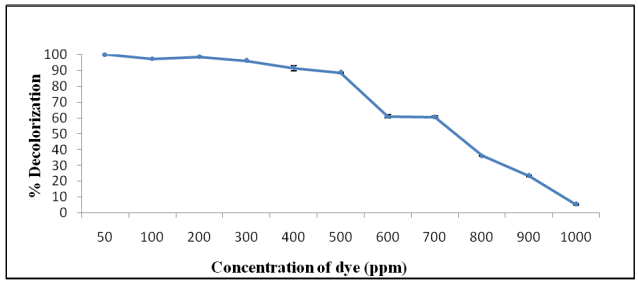

Fig. 8: Optimization of dye concentration for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

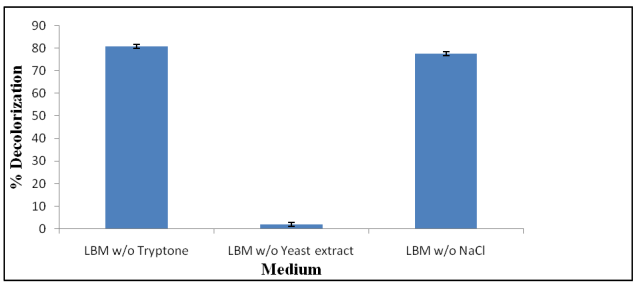

Fig. 9: Deletion assay of LBM for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

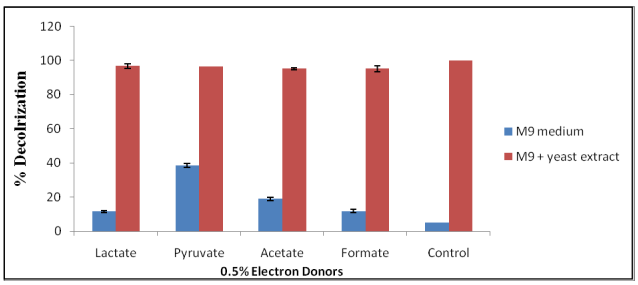

Fig. 13: Effect of electron donors (organic acids) on decolourisation of RR-195 by *E. casseliflavus* RDB_4.

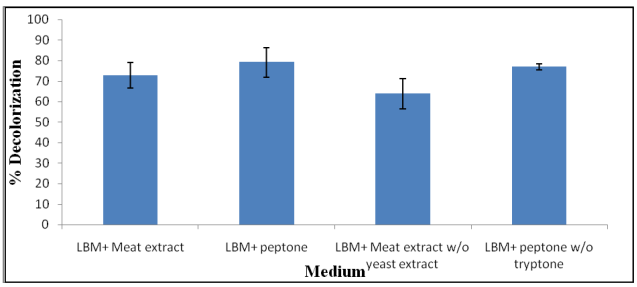

Fig. 10: Addition and substitution assay of LBM for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

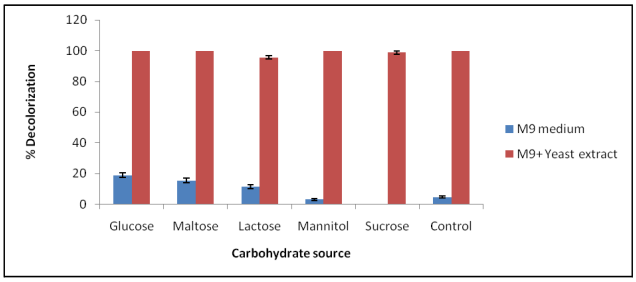

Fig. 14: Effect of electron donors (sugars) on decolourisation of RR-195 by *E. casseliflavus* RDB_4.

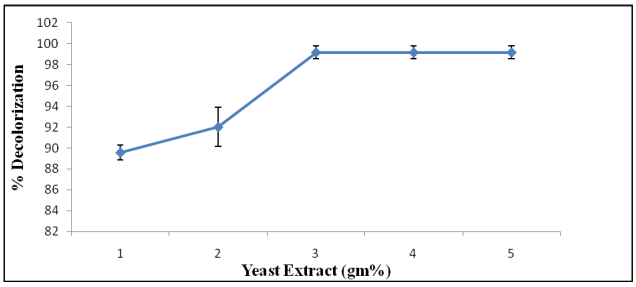

Fig. 11: Optimization of yeast extracts concentration for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

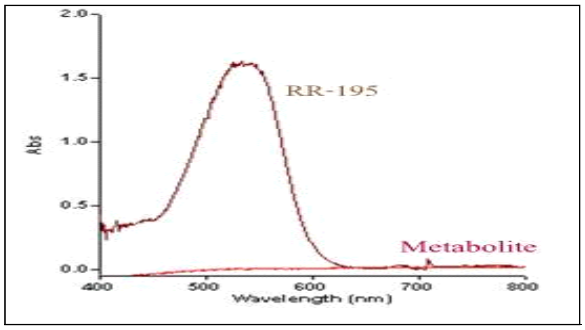

Fig. 15: Variation in UV-Vis spectra of RR-195 before and after decolourisation by *E. casseliflavus.*

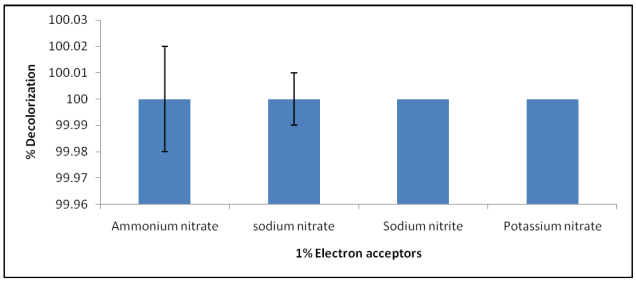

Fig. 12: Effect of inorganic nitrogenous compounds as electron acceptors for decolourisation of RR-195 by *E. casseliflavus* RDB_4.

reported 37°C as an optimal temperature for dye decolourisation by bacterial isolates (Kolekar et al. 2008) as well as by bacterial consortium (Saratale et al. 2010).

The optimum pH of the medium is critical for the growth, metabolic activity and enzyme production required for the decolourisation process. Our study showed significant decolourisation activity in the pH range of 6.0 to 10 (Fig. 5) with maximum decolourisation (93.66%) observed at pH 7 in 10 h. Tolerance to high pH is an added advantage in dye decolourisation studies since the processing of textile dyes includes steps involving the use of alkaline conditions and high temperatures (Aksu 2003). Our observation is also in accordance with Bhatt et al. (2005), where *E. coli* and *Pseu-*

domonas luteola were reported to exhibit maximum decolourisation at pH 7. In another study, *E. faecalis* showed maximum decolourisation of RO-16 in the pH range of 5 to 8 (Sahasrabudhe & Pathade 2012). Similar studies showing optimum activity in the pH range of 6.0-10.0 have been reported by several authors (Chen et al. 2003, Guo et al. 2007, Kilic et al. 2007).

The dyeing industries use a high amount of salts in industrial dye baths to ensure fixation of dye to the cellulose fibres (Gupta et al. 1990, Zollinger 1991). Salt concentrations up to 15 to 20% have been measured in wastewaters from dyestuff industries. Sodium levels are also elevated when sodium hydroxide is used in the dye bath to increase the pH. These high concentrations of sodium generally suppress microbial growth at levels above 3g% (Panswad & Anan 1999, Khalid et al. 2008). Hence, tolerance to high salt concentration for decolourisation studies is an essential requirement. In our study, *E. casseliflavus* RDB_4 showed significant decolourisation in LBM containing NaCl concentrations ranging from 1% to 5% with maximum decolourisation (100%) observed at 1% NaCl concentration (Fig. 6). In a recent study carried out on the detoxification of Direct black G, an azo dye, a thermophilic bacteria was also reported to tolerate salinity levels up to 5g% (Chen 2018).

The current study also reported that the decolourisation of RR-195 by *E. casseliflavus* RDB_4 was sensitive to oxygen since the optimum activity was observed under static conditions (Fig. 7). It may be due to the anoxic nature of *E. casseliflavus* RDB_4 which is a facultative anaerobic bacterium. Similar findings were also reported by other authors (Pearce et al. 2003, Sahasrabudhe & Pathade 2012). Bacterial degradation of azo dyes under oxygen-limiting conditions can be attributed to its strong reductive nature (Wuhrmann et al. 1980, Zimmermann et al. 1982, Banat et al. 1996, Chen et al. 2003). Since oxygen is a preferred electron acceptor in a bacterial system, it can be suggested that the dominance of aerobic respiration under agitation conditions may deprive the azoreductase enzymes of getting reduced by NADH, and hence minimize the decolourisation process (Stolz 2001, Kaushik & Malik 2009, Hu 1998).

The decolourisation potential of *E. casseliflavus* RDB_4 for RR-195 dye showed variations at different initial dye concentrations as indicated in Fig. 8. Complete decolourisation was observed in the concentrations ranging from 50 to 200 ppm and over 90% decolourisation was observed up to a 500 ppm concentration of RR-195 dye within 10 h. The natural environment contaminated with dyes and other toxic agents may possess higher concentrations of diverse dyes making it difficult for the bacteria to survive in such a strenuous environment. Inhibition of microbial cell growth due to interference with the synthesis of nucleic acids has been reported at higher dye concentrations (Shah & Patel 2014). Our study thus offers challenging application of *E. casseliflavus* RDB_4 for bioremediation of polluted dye effluents. Similar to our findings, Georgenia sp. CC-NMPT-T3 and an unidentified microflora have also shown decolourising ability up to 200 ppm of RR-195 dye and 600 ppm of Direct black G azo dye respectively (Sahasrabudhe & Pathade 2012, Chen et al. 2018).

Figs. 9 and 10 indicate the effect of deletion, addition and substitution assay to determine the effect of media components on the decolourisation potential of RR-195 by *E. casseliflavus* RDB_4. The deletion assay carried out, indicated the absolute necessity of yeast extract in the dye degradation process. In contrast, there was no significant difference observed in the dye degradation process in absence of NaCl and tryptone. The substitution of nutrient sources like yeast extract and tryptone from LBM with meat extract and peptone respectively, showed a decrease in decolourisation activity. Also, the addition of extra nutrient sources (meat extract and peptone) to LBM did not show any increased activity. All the above observations clearly indicate the positive role of yeast extracts for dye decolourisation processes. Hence, in further studies, the optimization of yeast extract concentration in the M9 medium was carried out. It was observed that 3% yeast extract was capable of decolourising 50 ppm RR-195 dye in 4 h as indicated in Fig. 11. Thus, yeast extract was supplemented in the minimal medium for all the further investigations. In contrast to the current study, our previous study reported a significant role of tryptone in addition to yeast extract for the decolourisation process (Birmole et al. 2014). Increased decolourisation in presence of yeast extract has also been reported for decolourisation of RR-195 by Georgenia sp. CC-NMPT-T3 (Sahasrabudhe & Pathade 2012) and other azo dyes by *P. Chrysogenum* (Praveen & Bhat 2012). Many investigators have reported yeast extract (50 $g.L^{-1}$) to be the most effective carbon and nitrogen source for the decolourisation of azo dye (Hosono et al. 1993, Lie et al. 1996). Dyes are deficient in carbon content and biodegradation without any extra carbon source is difficult (Coughlin et al. 1999).

In addition to various physical parameters, we also checked the effect of several electron acceptors for decolourisation of RR-195 by *E. casseliflavus* RDB_4. Complete decolourisation of RR-195 dye was observed in presence of various nitrates and nitrites added to M9 medium supplemented with 3% yeast extract (Fig. 12). Our results are not in agreement with those published by other researchers who have evaluated the interference of nitrate with azo

dye biodegradation. Thus, the bioremediation using *E. casseliflavus* RDB_4 has an advantage over other biological systems as this organism can decolourise RR-195 in the presence of nitrate and nitrite salts. Nitrate is used as an electron acceptor for anaerobic growth and is a potent regulator of the enzymes required for respiration using other electron acceptors (Pearce et al. 2006). Decolourisation is an oxidation-reduction process in which azo dyes serve as electron acceptors. Sodium nitrate is one of the typical salts included in the dye baths for improvement of dye fixation to the textile fibres and concentrations can reach 40-100 g dm^{-3}. Thus, nitrates can be present in dye wastewater and may, therefore, play a role in reducing the effectiveness of dye reduction; potentially down regulating azo reduction activity or acting as an electron sink in preference to azo bonds. Thus, in many other biological treatment systems colour removal is much slower when nitrate and nitrites are present in the system, with rapid denitrification preceding dye reduction, which suggests that the nitrate can compete with the dye for reducing equivalents by the biocatalysts (Dubin & Wright 1975, Pearce et al. 2006, Radhika et al. 2014).

Various organic acids (Fig. 13) and carbohydrates (Fig. 14) incorporated in the M9 medium were also checked for their potential to act as an electron donor for RR-195 decolourisation by *E. casseliflavus* RDB_4. The results indicated that none of the provided organic acids and carbohydrates could act as an electron donor. *E. casseliflavus* RDB_4 required yeast extract as the carbon and nitrogen source, and also as the electron donor for decolourising RR-195. Addition of carbon sources seemed to be less effective in promoting the decolourisation process, probably due to the preferences of the cells in assimilating the added carbon sources over the dye compound as the carbon source (Sahasrabudhe & Pathade 2012).

Mode of decolourisation: Investigations show that remediation of dyes by bacteria could be due to adsorption or biodegradation (Shah & Patel 2014). In our study, the non-autoclaved media containing live cells showed decolourisation and the autoclaved media containing dead cells did show decolourisation. The optimization experiments also suggested that the decolourisation process is dependent on various physical and biochemical factors which would not be the case if it was due to adsorption. Hence, it can be confirmed that the decolourisation of RR-195 dye is due to the metabolic process of *E. casseliflavus* RDB_4.

Effect of repeated dye decolourisation in a fed-batch process: The use of microbial isolates for commercial applications is relevant only if it can be used repeatedly in the continuous culture system. In our study, *E. casseliflavus* RDB_4 showed the ability to decolourise repeated addition of RR-195 dye aliquot (50 ppm) in M9 medium containing 3% yeast extract under a static condition. It exhibited the potential to completely decolourise the dye up to the third cycle. The first two cycles were complete within 2 and 3 h respectively, whereas the third cycle took 15 h for complete decolourisation. The eventual cessation of decolourisation was likely due to nutrient depletion (Kalyani et al. 2008, Sartale et al. 2009).

Immobilization: The process of immobilization of bacterial cells in calcium alginate also offers advantages of continuous processing of dyes in industrial wastes. This is because the process prevents washout of cells and allows high cell density to be maintained in the beads. Immobilization of cells in calcium alginate provides both aerobic and anaerobic conditions for cells to act on dyes which efficiently degrade textile dyes. Unfortunately, the immobilization of cells in our study did not support efficient decolourisation of RR-195 dye. It took 24 h for immobilized cells to decolourise 50 ppm of RR-195, whereas free cells of *E. casseliflavus* strain RDB_4 decolourised RR-195 within 4 h in the M9 medium. Immobilized cultures tend to have a higher level of activity and are more resilient to environmental perturba-tions with respect to pH or exposure to toxic chemicals. In our study, the inefficiency of the immobilized cells might be due to the anoxic condition produced in the beads which could not support the metabolism of the organism and therefore decolourisation of the dye.

Ecotoxicity studies: Table 1 represents the observed effects of RR-195 dye and its degraded metabolites on germination of *P. mungo* and *T. aestivum* seeds. It clearly reflects the toxic effect of untreated RR-195 dye as indicated by the stunted radicle and plumule length as compared to control.

Table 1: Ecotoxicity study of RR-195 and its degraded metabolites by *E. casseliflavus* RDB_4.

Plant name	Average length in cm					
	Control		RR-195		Metabolites	
	Radicle	Plumule	Radicle	Plumule	Radicle	Plumule
P. mungo	3.3	1.8	0.4	0.2	3	1.6
T. aestivum	2.7	1.2	0.3	0.2	2.5	1.2

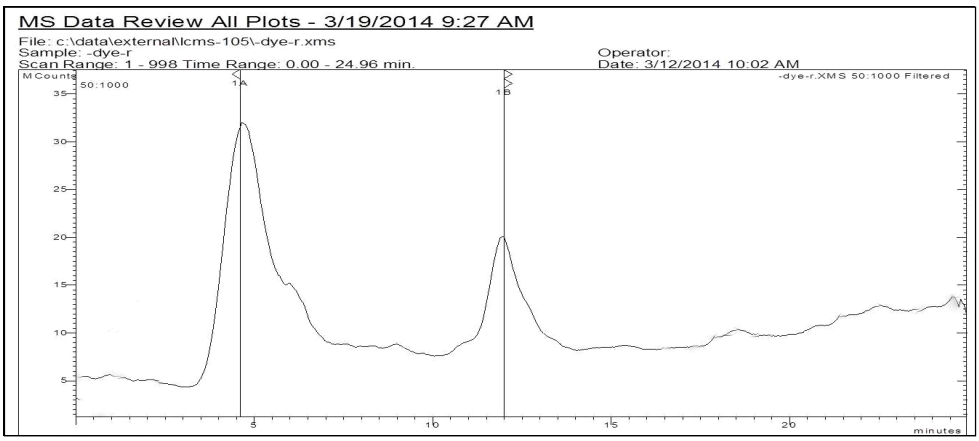

Fig. 16: HPLC chromatogram of RR-195 dye.

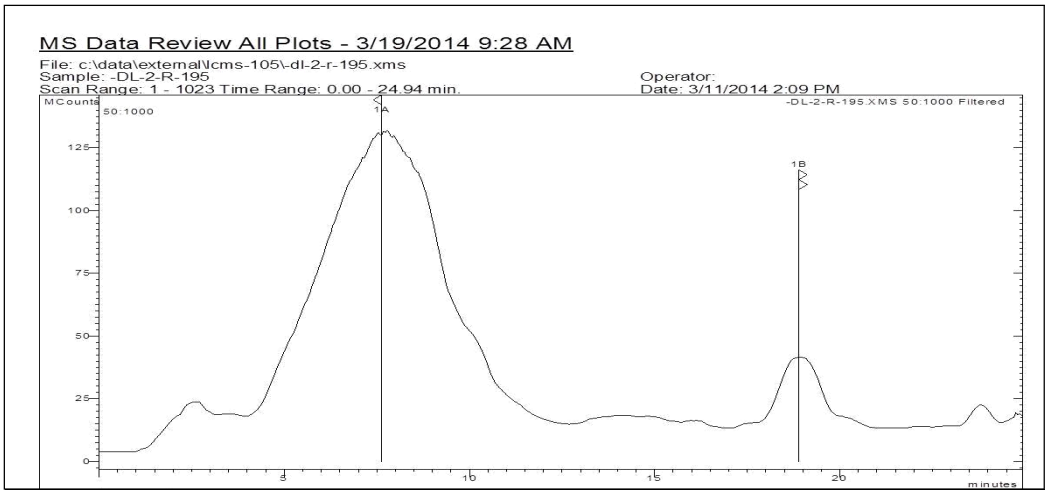

Fig. 17: HPLC chromatogram of degraded RR-195 dye metabolites.

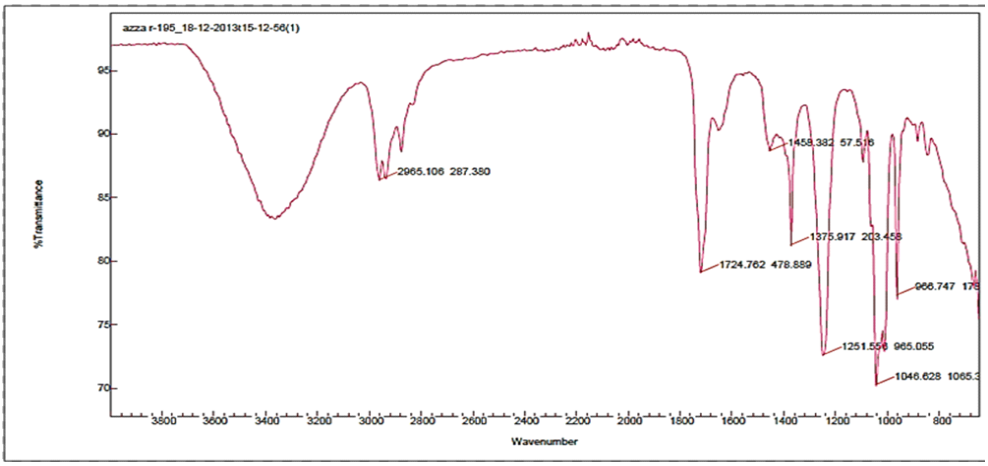

Fig. 18: FTIR spectrum of RR- 195 dye.

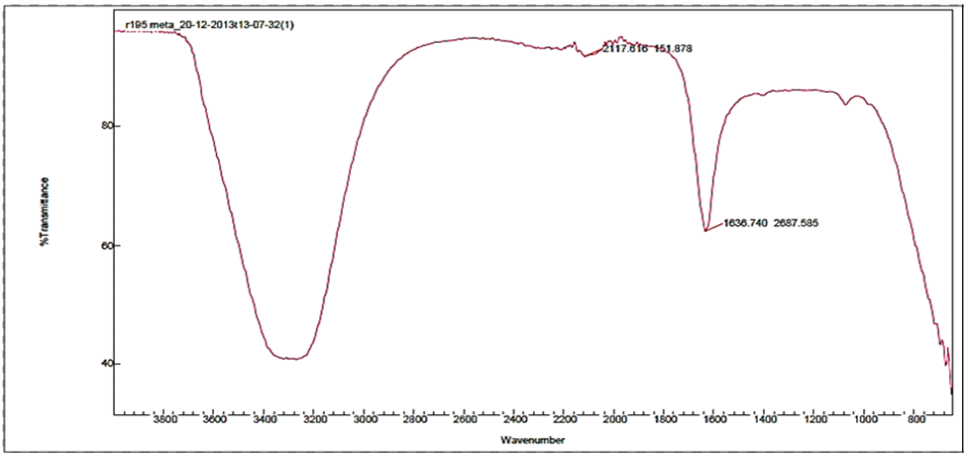

Fig. 19: FTIR spectrum of degraded RR-195 dye metabolites.

The degraded metabolites, on the other hand, showed no growth inhibition indicating that the biodegraded metabolites of RR-195 are non-toxic.

Dye effluents contain diverse contaminants, which when discarded into water-bodies, may cause serious environmental and health hazards. Unfortunately, in spite of the known hazards, untreated dye effluents commonly find their way into neighbouring water resources including rivers and lakes and ultimately into agricultural fields. Our study indicated that the metabolites obtained after degradation of RR-195 dye are nontoxic to the germination of the seeds. This opens a new dimension for application of our test isolate i.e. *E. casseliflavus* RDB_4 for treatment of industrial dye RR-195. Similar studies have shown effective degradation of azo-dyes by *Pseudomonas aeruginosa* (Elfarash et al. 2017) and *Staphylococcus hominis* subsp. *hominis* DSM 20328 (Parmar & Shukla 2017). A phytotoxicity assay carried out using *Vigna radiata* also indicated effective detoxification of AB dye. In the same study, the genotoxicity assay with *Allium cepa* showed that pure AB dye solutions significantly reduced mitotic index (MI) and induced various chromosomal abnormalities, whereas, bioremediated dyes induced relatively less genotoxicity in nature (Haq et al. 2017).

Bio-decolourisation and biodegradation analysis: The UV-Vis spectral analysis of RR-195 dye showed a decrease in absorbance from 1.161 to -0.1210 at 540 nm after the biodegradation process (Fig. 15). The decrease in absorbance or disappearance of the absorbance peak at the maximum wavelength of the dye is indicative of biodegradation due to microbial metabolism (Novotny 2004). In order to confirm complete degradation of dye, the cell pellet was recovered to further extract the adsorbed dye if any, using methanol and subjected to UV- Vis Spectrophotometric analysis. It showed no absorbance at 540 nm, hence confirming complete degradation.

Figs. 16 and 17 indicate the HPLC elution profile of RR-195 and its degraded metabolites respectively. Both chromatograms showed different retention times. RR-195 dye showed major peaks at retention time 4.598 min and 11.988 min, whereas degradation products showed major peaks at retention time 7.629 min and 18.892 min. The disappearance of peaks in the dye chromatogram and occurrence of new peaks in the metabolite chromatogram clearly indicates successful biodegradation of RR-195 dye.

A noticeable difference was also observed between FTIR spectra of RR-195 and its metabolites (Figs. 18 and 19) further implying the biodegradation of RR-195 by *E. casseliflavus* RDB_4. Table 2 represents the probable functional groups of the peaks observed in the FTIR spectrum of the RR-195 dye and its degraded metabolites. The peak of sulfide and sulphone (S=O bond) stretching at 1375.917,

Table 2: Probable functional groups of the peaks observed in FTIR spectrum of RR-195 dye and its degraded metabolites.

Wave number	Probable functional group
RR-195 dye	
2965.108	Asymmetric aliphatic C-H
1724.762	C=O ketone group
1458.382	N=N stretching of the azo group
1375.917	S=O stretching of SO3
1251.556	The C-N stretching vibration of aromatic primary amine
1046.628	C-O or C-N
Degraded metabolites	
2117.616	Amines
1636.740	C=C stretching indicating aromatic nature

Table 3: Decolourisation of different textile dyes by *E. casseliflavus* RDB_4.

Name of the dye	λ max(nm)	% Decolourisation
Dispersive blue B3	634	25.7
Reactive brilliant blue-198	628	35.08
Reactive brilliant blue-19	600	54.69
Reactive red-120	51	89.08
Direct red 5B	507	100
Reactive orange-16	495	95.63
Reactive black-5	600	100
Coracion green HE4B	652	98.27
Blue MEBRF 150%	624	100
Navy blue ME2G	611	100
Navy blue MEBL	617	100
Reactive yellow	425	100
Coracion red HE3B-N	415	100
Coracion orange HE2R	491	100
Orange ME2R	491	45.38
Corafix red ME4B	541	91.50

Table 4: Decolourisation of different dye mixtures by *E. casseliflavus* RDB_4.

Dye mixture	λ max(nm)	% Decolorization
Navy blue MEBL + RR-195	552	100
Direct red 5B + RR-195	541	98.64
Reactive red-120 +RR-195	527	97.91
Reactive black -5 + RR-195	551	80.69
Coracion green HE4B + RR-195	551	91.10
Blue MEBRF 150% + RR-195	552	100
Navy blue ME2G + RR-195	551	98.06
Reactive orange - 16 + RR-195	521	98.45
Reactive brilliant blue -19 + RR-195	540	74.65
Reactive yellow + RR-195	545	100
RR-195+ Navy blue MEBL + Reactive Red-120 + Reactive yellow	552	100

1251.558 and 1045.628 was observed in control dye spectrum. These stretching peaks were completely absent in the metabolite spectra indicating a breakdown of S=O bond in biodegradation of the RR-195 dye. The peak of the azo bond (N=N) at 1458.382 was observed in the control dye spectrum but was completely absent in the metabolite spectra indicating a breakdown of N=N bond in the RR-195 dye. The breakdown of asymmetric aliphatic –CH, C-O, C-N and C-H groups was also observed in the metabolite spectra. The metabolite spectra also showed the presence of amines which must have been produced during the biodegradation process of the dye.

The different chemical structures in azo dyes have a prominent effect on the decolourisation and biodegradation rate (Wuhrmann et al. 1980, Mendez et al. 2005). Depending on the number and placement of the azo linkages, some dyes are biodegraded more rapidly than others. In general, more the number of azo linkages, the slower is the rate of degradation. In this effect, Brown & Laboureur (1983) have reported that two poly-azo dyes showed very low decomposition rate when compared to dyes having four mono azo and six diazo linkages. Fibre-reactive azo dyes often have side groups responsible for solubilization, along with reactive groups being nucleophilic in nature. Depending on the type of these substituents, biodegradation rate might be changed.

Decolourisation of various textile dyes and mixture of dyes: Synthetic dyes of different structure are often manufactured by industries, and therefore, the effluents are markedly variable in composition. Due to this reason, there is a need for screening of cultures which possess the ability to decolourise a range of dyes and even mixtures of several dyes. Our study showed that *E. casseliflavus* RDB_4 could decolourise twelve azo-dyes in addition to RR-195 and also methylene blue and Congo red in M9 medium containing 3% yeast extract within 5 h. Amongst azo-dyes, *E. casseliflavus* RDB_4 showed complete decolourisation (100%) of 8 dyes under static condition, and above 90% decolourisation was observed for 5 other dyes (Table 3). In addition, it also showed considerable decolourisation ability towards the mixture of two to four dyes within 5 h (Table 4). Chen et al. (2003) have reported significant decolourisation for mixtures of dyes within two days by *Aeromonas hydrophila*. *E. faecalis* YZ66 had the ability to decolourise mixtures of various dyes such as RR-195, Direct Red 81, Acid blue 113, Reactive orange 16 and Reactive yellow 145. The rate of decolourisation was very fast and colour removal was 80.58% within 2 h (Sahasrabudhe & Pathade 2012).

CONCLUSIONS

The RR-195 dye is a sulphonated azo-dye used extensively in the textile industries and discharged in the environment, thus compromising the well-being of its inhabitants. Our study offers a solution to this problem by exploiting the capability of *E. casseliflavus* RDB_4 to degrade RR-195 into nontoxic compounds. The tolerance of *E. casseliflavus* RDB_4 to high salt and dye concentrations in alkaline conditions makes it a perfect candidate for bioremediation of industrial dye wastes.

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