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Bacterial Decolourization & Degeradation Of Reactive Red 35: A Brief Treatise.

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Abstract

Environmental pollution is one of the major and most important problems of modern world. Increasing industrialization for fulfilling the need and demand of overgrowing human population lead to the production of environmental pollutants. These pollutants are released into the environment and causes detrimental effects on the humans, plants, animals, microbes and ecosystem integrity. We must focus our attention on ways for the eradication and reduction of pollution (Hazrat, 2010). The textile industry is the one of the major greatest generators of liquid effluent pollutants, due to high quantities of water used in the dyeing processes. It is estimated that 2,80,000 tons of textile dyes are discharged in such industrial effluents every year worldwide (Saratale *et al*.,2011). Azo dyes are the most widely used dyes and represent over 60-70% of the total dyes (Solis *et al*., 2012). Azo dyes in general can be defined as ones which have, chromophoric azo group (-N=N-) attached to an aromatic or heterocyclic nucleus at one end and an unsaturated molecule of carbocyclic, heterocyclic or aliphatic type at the other end. These dyes are found to be largest classification of dyes with the Color Index (CI) of more than 10,000 (Ghaly *et al*., 2014). During the dying process all the dye does not bind to the fabrics; and release (2-50%) in effluent. The existence of azo dyes and its intermediates in aqueous ecosystems leads to aesthetically unacceptable coloration of waters, and reduced sunlight penetration, photosynthetic

activity, dissolved oxygen leveland consequently leading to death and putrefaction of aquatic animals (Agrawal *et al*., 2014). Therefore, treatment of textile wastewater is necessary prior to discharge.

Traditional treatment methods utilized for treatment of textile wastewater are having several limited applications and drawbacks. Biological treatment methods appear to be a promising technology for treatment of textile wastewater in eco-efficient manner. Uses of microbial technology for treatment of textile wastewater have several advantages, such as environmentally friendly, cost competitive, producing less sludge, efficient mineralization in less toxic or non-toxic compounds with combination of different treatment and less input of chemicals and water for treatment (Christian *et al*., 2005; Hazrat, 2010; Solis *et al*., 2012).

The effectiveness of microbial treatment of textile wastewater lies on the ability of the selected microorganisms to decolorize and degrade the dye present in it. Microbes acclimatize themselves to the toxic pollutants and develop natural resistance and transform them into less harmful forms (Saratale *et al*., 2011). A wide variety of microorganisms, including bacteria, fungi, yeasts, actinomycetes, and algae are capable for decolorization and degradation of dyes (Agrawal *et al*., 2014; Pandey *et al*., 2007). Under facultative and anaerobic conditions, bacterial degradation of azo dyes is initiated generally by cleavage of azo bonds resulted in the formation of colorless aromatic amine and its derivatives, which are mutagenic and toxic and cannot be metabolizedeasily under the same conditions under which they produce. They are generally degraded under aerobic condition (Balapure *et al*., 2014). So combination of different treatment methods must be exploited for effective biodegradation of textile wastewater. Thus, the bacterial population employed in a treatment of dye containing wastewater must be able to work under both anaerobic/microaerophilic and aerobic conditions to obtain efficient degradation of azo dyes (Balapure *et al*., 2014). Utilization of synergistic effect of mixed bacterial population for treatment of textile wastewater has advantages of effective mineralization of dyes (Jain *et al*., 2012).The immobilization of microbial cultures on inert carrier materials in bioreactor for large scale treatment of textile wastewater has widened their applications and improved their performance, stability against higher organic loading rate (Balapure *et al*.,2015; Lade *et al*., 2015).

Thus, in a light of the above discussion and need to develop an efficient method for the treatment of textile wastewater with potent microbial cultures,different potential bacteria having the capability to decolorize and degrade the textile dyes were isolated and their performance were enhanced by optimizing process parameters.Potent bacterial isolates were also applied for reactor scale study for the treatment of textile wastewater. Briefly, the entire study for microbial decolorization of azo dyes was aimed with the following objectives:

- Screening and isolation of bacteria capable of decolorizing azo dyes.
- Identification and characterization of azo dye decolorizing bacteria.
- Assessment of ability of potent bacterial isolates to decolorize different azo dyes.
- Assessment of dye decolorization performance of potent bacterialisolates in mixture with

other isolates.

- Optimization of physico-chemical parameters to increase dye decolorization performance of bacterial isolates.
- Determination of enzyme responsible biodegradation of dye.
- Study the biodegradation mechanism of dye.
- Reactor scale study for treatment of azo dye containing textile wastewater.
- Toxicity assessment of treated dye and textile wastewater.

To fulfill above objectives, present research work was initiated with the isolation and screening of dye decolorizing bacteria.Reactive Red 35 (RR35), a vinyl sulphone based monoazo dye, widely utilized in textile dyeing was selected as a model dye for study. Fifteen different soil/ sludge and wastewater samples, from dyecontaminated siteswere collected. From these samples total eighty-three different bacteria were isolatedby enrichment culture technique using Bushnell Hass medium (supplemented with 2.5 g/L yeast extract and 100 mg/L of RR35). These bacterial isolates were screened for their RR35 dye decolorizing ability at 100 mg/L concentration, and five potent bacterial isolates (showing >70% dye decolorization) were selected for further study and characterized by cultural, morphological and with different biochemical tests. They are identified with 16S rRNA sequence, as *Pseudomonas aeruginosa* ARSKS20,*Enterococcus gallinarum* NRK, *Pseudomonas aeruginosa* PKS, *Bacillus cereus* AJS1,and *Lysinibacillus macroides*ARS5and the gene sequence were submitted to GenBank at NCBI with GenBank Accession number JN817386.1,KC814158.1,KR706564, KR706562 and KR706563, respectively. Antibiotic susceptibility testing of these five potential bacterial isolates were also carried out. They are sensitive towards wide spectrum range of antibiotics.

Each of these five bacterial isolates individually and in combination with other pure cultures (among five) were tested for their RR35 dye decolorization ability. Individually, pure cultures of *P. aeruginosa* ARSKS20,*E. gallinarum* NRK, *P. aeruginosa* PKS, *B. cereus* AJS1, and *L. macroides*ARS5were showing83.02, 88.88, 72.23, 70.66 and 71.98% decolorization of RR35 at 100 mg/L concentration, respectively, after 24 h at 35±2°C, under static condition. The bacterial mixture of two best potent isolates*P. aeruginosa* ARSKS20 and *E. gallinarum* NRK (designated as MBR1) showed 95.54, 94.02 and 92.19% decolorization of 100, 300 and 500 mg/L of RR35 respectively, after 24 h of incubation. The bacterial mixture of *P. aeruginosa* ARSKS20, *E. gallinarum* NRK, *B. cereus* AJS1and *L. macroides*ARS5(designated as MBR2) showed 99.56, 98.09 and 97.53% decolorization of 100, 300 and 500 mg/L of RR35 respectively, after 24 h of incubation. Thus, nearly complete decolorization was observed with the bacterial mixture-MBR2 even at 500 mg/L RR35 concentration, it might be due to co-metabolism among bacteria, which was of major interest for large scale treatment of textile effluent.

Two most potential cultures *P. aeruginosa* ARSKS20 and *E. gallinarum* NRKwere also assessed for their ability to decolorize different azo dyes at 100 mg/L concentration. The results

suggest that *P. aeruginosa* ARSKS20 can decolorize thirteen different azo dyes, while *E. gallinarum* NRK can efficiently decolorizeeleven different azo dyes. Both potent isolates decolorized RR35 at fastest rate than the other dyes. Thus, further detailed investigation for decolorization and degradation dye was carried out with of RR35 dye by pure cultures of *P. aeruginosa* ARSKS20 and *E. gallinarum* NRK.

Optimization of physico-chemical parameters for RR35 dye decolorization by *P. aeruginosa* ARSKS20 was carried out with one parameter at a time approach. Dye decolorization performance of *P. aeruginosa* ARSKS20 was assessed under various environmental parameters such as static *vs* shaking condition (120 rpm), initial dye concentrations (50–500 mg/L), pH values (4–11) and temperatures (25–50˚C), inoculum size (1-10%, v/v) and in the presence of different supplemental carbon and nitrogen sources. *P. aeruginosa* ARSKS20 decolorized RR35 only under static condition. It shows maximum 95.11% decolorization of RR35 at a rate of 8.27 mg/L/h at 40˚C, pH 8, under static condition at 100 mg/L concentration. 5% (v/v) inoculum volume of *P. aeruginosa* ARSKS20was proved as an optimum for RR35 dye decolorization. *P. aeruginosa* ARSKS20 showedthe obligatory requirement of yeast extract for RR35 decolorization and utilized yeast extract as both carbon and nitrogen source. It can tolerate upto 50 g/L NaCl concentration and shows 90% dye decolorization at a rate of 2.41 mg/L/h.The decolorization of repeated addition of dye aliquots (100 mg/L) to culture media was also studied. Under optimized conditions, it could efficiently decolorize RR35 with increasing rate upto twelve repeated dosing without supplementation of new nutrients. Growth and dye decolorization ability of *P. aeruginosa* ARSKS20 is strongly affected in presence of metals. Inpresent study effect of chromium, nickel, cobalt and copper were assessed. Growth and dye decolorization ability of *P. aeruginosa* ARSKS20were adversely affected at 1.9 mM of Cr(VI), 7.5 mM of Ni(II), 6.5mM of Co(II) and 0.5 mM of Cu(II).

The enzyme profile study of*P. aeruginosa* ARSKS20 during RR35 decolorization, shows significant induction in the activities of laccase (20%), lignin peroxidase (24%), tyrosinase (106%), veratryl alcohol oxidase (260%), NADH–DCIP reductase (33%), and azoreductase (139%)enzymes suggest their active involvement in RR35 dye decolorization. Decolorization and degradation of RR35 by *P. aeruginosa* ARSKS20was demonstrated through UV-visible spectral scanning, HPLC, HPTLC, and FTIR analysis. The biodegradation mechanism of RR35 by *P. aeruginosa* ARSKS20is predicted with the help of GC-MS analysis. The GC-MS analysis of metabolites obtained after decolorization suggest, RR35 is degraded into 1-amino-2-hydroxy-5-[2-(sulphooxy ethyl)] benzene, 1-amino-2-methoxy-5-[2-(hydroxy)ethyl] sulphonyl benzene, 1-amino-2-methoxy benzene, 2-amino-8-(acetylamino) naphthalen-1-ol, naphthalene-1-7-diamine, 2-amino naphthalen-1-ol and naphthalene.

The toxicological studies of metabolites obtained after RR35 degradation by *P. aeruginosa* ARSKS20 were performed with various techniques, viz. microbial toxicity with*Bacillus subtilis* (MTCC 1305) and *Azotobacter chroococcum* (MTCC 7724); cytogenotoxicity, comet assay, antioxidant enzyme status, protein oxidation, lipid peroxidation with *Allium cepa* assay; and

phytotoxicity with *Sorghumbicolor, Triticum aestivum* and *Phaseous mungo.* The toxicity studies collectively conclude the non-toxic nature of metabolites obtained after degradation of RR35. Thus, *P. aeruginosa* ARSKS20 candecolorize and degrade RR35 dye efficiently into non-toxic low molecular weight compounds and it can be further utilized for eco-friendly treatment of textile wastewater at larger scale.

Optimization of physico-chemical parameters for RR35 dye decolorization by *E. gallinarum* NRKwas carried out with one parameter at a time approach.Dye decolorization performance was assessed under various environmental parameters such as static *vs* shaking condition, initial dye concentrations (50–500 mg/L), pH values (4–11), temperatures (25–50˚C) and in presence of different supplementalcarbon and nitrogen sources. *E. gallinarum* NRKshowed very poor dye decolorization under shaking condition. It showed maximum 96% decolorization of RR35 at a rate of 76.81 mg/L/h at 40˚C, pH 7, under static condition at 300 mg/L RR35 concentration.Yeast extract at 5 g/L concentrations (with 5% (v/v)inoculum) was proved as an optimum concentration for RR35 dye decolorization. Isolate could efficiently decolorize RR35 (85.38%) at higher salinity (40 g/L) with a rate of 68.30 mg/L/h. The dye decolorization capacity of *E. gallinarum* NRK was evaluated with repeated spiking of 300 mg/L RR35 dye. Under optimized conditions, it could efficiently decolorize RR35 with increasing rate upto repeated dosing without supplementation of new nutrients.

The enzyme profile study of*E. gallinarum* NRKduring the RR35 decolorization showedsignificant induced activities of intracellular laccase (35%), extracellular laccase (438%), intracellular tyrosinase (75%), extracellular tyrosinase (226%), lignin peroxidase (Lip) (75%), veratryl alcohol oxidase (43%), NADH–DCIP reductase (25%), and azoreductase (418%) enzymes.

Decolorization and degradation of RR35 by *E. gallinarum* NRKwas demonstrated through UV-visible spectral scanning, HPTLC and FTIR analysis. The biodegradation mechanism of RR35 by *E. gallinarum* NRKis predicted with the help of GC-MS analysis. The GC-MS analysis of metabolites obtained after decolorization suggest, RR35 is degraded into1-amino-3-[2-(sulphooxy)ethyl]sulphonyl benzene, 2-amino-8-(acetylamino)naphthalen-1-ol and naphthalene 1,7-diamine. Toxicological studies of metabolites obtained after RR35 degradation by *E. gallinarum* NRK were performed with various techniques, viz. cytogenotoxicity, comet assayin*Allium cepa* and phytotoxicitywith *T. aestivum, P. glaucum, P. mungo* and *V. radiata.*The toxicity studies collectively conclude non-toxic nature of metabolites obtained after degradation of RR35.Thus, *E. gallinarum* NRK candecolorize and degrade RR35 dye very efficiently and it can be further appliedfor eco-friendly treatment of textile wastewater.

Scale-up study was carried out with microaerophilic downflow fixed film reactors and with sequential anaerobic-plugflow fixed film reactor/aerobic-airlift fixed film reactor. Furnace charcoal was used as packing materials for biofilm development.

For scale up study, textile waste water was collected from Global Dyeing and Printer,

Behrampura, Ahmedabad.This wastewater was collected during the dyeing of purple color shading on cotton sheets (cloths) for which they have utilized Reactive Red 35 (RR35) and Reactive Blue 160 (RB160) dyes for developing their purple color shade. Thus, collected wastewater was the residual mixture of Reactive Red 35, Reactive Blue 160 dyes and other ingredients utilized for dyeing. Collected textile wastewater was analyzed for its physico-chemical characteristics, and it showed alkaline pH (9.0 ± 0.4), high total solids (TS) and total dissolved solids (TDS)content with chemical oxygen demand (COD) and biological oxygen demand (BOD) value of 1920 ± 250 mg/L and 495 ± 85 mg/L, respectively.

The microaerophilic treatment of textile wastewater was carried out with the establishment of two different microaerophilic downflow fixed film reactors (DFFR). One microaerophilic Downflow fixed film reactor (DFFR1) was established with biofilm formation of active mixed culture MBR1, which contains mixed bacterial cultures of *P. aeruginosa* ARSKS20 and *E. gallinarum* NRK. The second microaerophilic downflow fixed film reactor (DFFR2) was established with biofilm formation of active mixed culture MBR2, which containedbacterial cultures of *P. aeruginosa* ARSKS20, *E. gallinarum* NRK, *B. cereus* AJS1, and *L. macroides*ARS5 (isolated during the initial phase of this study).Both microaerophilic DFFR1 and DFFR2 were operated with organic loading rate (OLR) of 0.088-2.2 kg COD/m^3/d after development of active biofilm.Maximum ADMI (American Dye Manufacturer's Institute) removal 95.45 and 96.02% observed in DFFR1 and DFFR2, respectively, with OLR of 0.44 kg COD/m^3/d. BOD removal in DFFR1 and DFFR2 were obtained in the range from 90-64.03% and 91.33-74.24%, respectively. COD removal in DFFR1 and DFFR2 was obtained in the range from 79.88-59.55% and 83.68-64.89%, respectively. Alkalinity and pH were increased with increasing OLR. . Maximum TS and TDS removal were 67.21 and 68.13%; and 68.52 and 70.78%, respectively obtained in DFFR1 and DFFR2 at 0.264 kg COD/m^3/d OLR. Higher induced enzyme activities of azoreductase, veratryl alcohol oxidase, tyrosinase and lignin peroxidase were observed in DFFR2 in comparing to DFFR1. Moderately higher induced activities of NADH-DCIP reductase was observed in DFFR1 than DFFR2. Degradation of dyes present in textile wastewater was confirmed through UV- visible spectral scanning, HPTLC and FTIR analysis. With the help of GC-MS analysis probable degradation mechanism of RR35 and RB160 dyes (present in textile wastewater) during microaerophilic treatment are elucidated. The formation and disappearance of different metabolites of RR35 and RB160 during different HRT of DFFR1 and DFFR2 were studied with GC-MS analysis. Results suggest that the intermediates formed from the initial cleavage of RR35 and RB160 were detected during 1 d and 2 d HRT, and sequentially formed lower molecular weight compounds and some complex compounds were detected during 3 d and 5 d HRT.

The toxicological studies of treated (with DFFR1 and DFFR2) textile wastewaters were carried out by phytotoxicity and their effect on soil fertility properties. Phytotoxicitystudies with *V. radiata, P. mungo* and *T. aestivum*conclude non-toxic nature of treated textile wastewater. Toxicological effects on soil fertility upon utilization of treated (with DFFR1 and DFFR2) and

untreated textile wastewater were assessed with various parameters, viz. soil pH, conductivity, N,P,K value, soil enzyme activity (acid phosphatase, alkaline phosphatase, dehydrogenase), the number of soil bacteria (amylase and lipase producing bacteria, and *Azotobacter* sp.). The overall results suggest that after treatment with DFFR1 and DFFR2, textile wastewatersare safe for disposal on land and utilization for irrigation purpose along with normal water.

The anaerobic and sequential anaerobic/aerobic treatment of textile wastewater was carried out with uniquely designed anaerobic-plugflow fixed film reactor(PFFR)/aerobic-airlift fixed film reactor(AFFR). Anaerobic-PFFR were operated 2-6 d hydraulic retention time (HRT) (OLR of 0.33-0.99 kg COD/m^3/d) and effluent of PFFR sequentially treated for more 2 d with aerobic-AFFR. Thus, combined anaerobic/aerobic treatment (with PFFR/AFFR) were operated collectively by 4-8 d HRT. Anaerobic treatment of textile wastewater showed 96-95% ADMI removal during OLR of 0.33-0.99 kg COD/m^3/d. During subsequent aerobic treatment, no remarkable ADMI removal was observed. COD removal during anaerobic and with sequential aerobic treatment was observed in range, from 80.14- 50.87%, and 84.37-64.06%, respectively. BOD removal during anaerobic and with sequential aerobic treatment was observed in range, from 84.51-72.54%, and 95.77-80.28%, respectively. The higher efficiency of sequential anaerobic/aerobic treatment was observed with higher OLR and at shorter retention time. Sequential anaerobic/aerobic treatment showed efficient removal of total solid and total dissolved solid even at increasing OLR. Decreased alkalinity was observed after sequential anaerobic/ aerobic treatment. It might be due to degradation of amine during subsequent aerobic treatment. During anaerobic treatment, maximum 23.08% methane content was observed during OLR of 0.49 kg COD/m^3/d and at 4 d HRT.

Anaerobic degradation of RR35 and RB160 present in textile wastewater were confirmed through UV- visible spectral scanning, HPTLC and FTIR analysis. The probable degradation mechanism of RR35 and RB160 dye during anaerobic treatment are elucidatedwith the help of GC-MS analysis. The formation and disappearance of different metabolites from degradation of RR35 and RB160 during different HRT (2, 3 and 6 d) of anaerobic PFFR were studied with GC-MS analysis. High molecular weight intermediates and intermediates formed duringthe initial cleavage of RR35 and RB160 were detected 2 d and 3 d HRT, and sequentially formed lower molecular weight compounds and some complex compounds were detected during 6 d HRT.

Sequential degradation of metabolites under aerobic treatment with AFFR was confirmed through UV- visible spectral scanning, HPTLC and FTIR analysis. Degradation of these metabolites under aerobic treatment (with aerobic-AFFR) was evaluated by comparing the metabolites those were formed during the 6 d HRT treatment with anaerobic PFFR, with the metabolites detected after its sequential treatment (for 2 d) under aerobic AFFR. Results show that initial total sixteen compounds are detected after 6 d anaerobic treatment with PFFR. From these only five compounds [(1)2-amino benzene- 1,4-disulphonic acid; (2)2,5-diamino benzene-1,4-disulphonic acid; (3)2-amino-8-(acetylamino) naphthalen-1-ol; (4)1-amino-3-[2-(sulphooxy) ethyl] benzene; (5)1-amino-3-[2-(sulphooxy)ethyl]sulphonyl benzene]are left out after subsequent

aerobic treatment with AFFR, and remaining eleven compounds may be transformed or degraded during aerobic treatment. Thus, sequential anaerobic/aerobic treatment of textile wastewater, provides very efficient mineralization of textile wastewater.

The toxicological evaluation of textile wastewater after anaerobic and sequential anaerobic/ aerobic treatment were performed by analyzing various parameters, viz. % seed germination (after 5 d), plumule, radical and fibril length, number of legume formation (after 50 d) during phytotoxicity testing with *V. radiata*. Results suggest the toxic effect of untreated textile wastewater was reduced after anaerobic treatment, which was further reduced very efficiently after sequential aerobic treatment.

From the present investigation following conclusions are derived:

- Pure cultures of *P. aeruginosa* ARSKS20 and *E. gallinarum* NRK can efficiently decolorize and degrade Reactive Red 35 dye and are also capable to decolorize wide spectrum of azo dyes.
- Mixed bacterial cultures of (1) *P. aeruginosa* ARSKS20 and*E. gallinarum* NRK;and(2) *P. aeruginosa* ARSKS20, *E. gallinarum* NRK, *B. cereus* AJS1, and *L. macroides*ARS5, can efficiently decolorize, degrade and detoxify textile wastewater with microaerophilic downflow fixed film reactor.
- Sequential anaerobic/aerobic treatment of textile wastewater with anaerobic plugflow fixed film reactor/aerobic airlift fixed film reactor can give a higher efficient treatment of textile wastewater with higher organic loading rate and efficiently reduced toxicity of textile wastewater and provide best eco-friendly remediation of textile wastewater.

Thus, microbial treatment of azo dyes and azo dyes containing textile wastewater is the most efficient eco-friendly treatment approach tobe utilized for larger scale treatment.

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