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Full Length Research Paper

Alkaliphilic degradation of mixed reactive dyes (C.I.RY 125 and C.I.RB 52) by a moderately alkaliphilic bacterial consortium – optimization and bench scale studies

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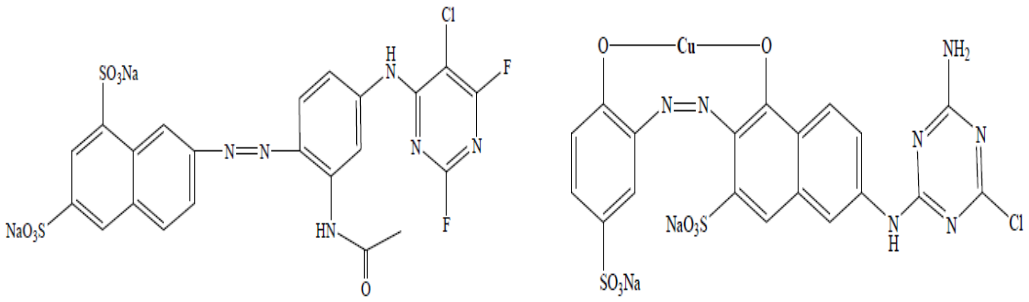
ABSTRACT

Harmful effects of textile industry effluents are a huge problem nowadays. The recalcitrant nature of modern synthetic dyes has led to the imposition of strict environmental regulations. There is a need to devise cost-effective processes to remove the colour from wastewater produced by the textile industry. Microbial decolorisation of the dyes has been proved to break down complex organic compounds into completely harmless state. Textile effluents contain toxic chemicals and have very acidic and alkaline organic contaminants. Currently enormous interest has been encouraged on the decolourisation of dyes by alkaliphilic bacterial strains. The main objective of the present work is to reduce the colour of the reactive dyes under alkaline conditions. The capabilities of bacterial consortium were explored for the decolorization of textile mixed reactive dyes (C.I.RY 125) and (C.I.RB 52). The decolorization of azo dyes was studied at various concentrations (100–500ppm). The maximum decolorization of reactive azo dyes (C.I.RY 125dye) and (C.I.RB 52dye) were 79.6% and 77.7% respectively. Also optimization and bench scale bioreactor studies were done to test the effect of parameters like pH, temperature, C and N sources on the COD removal efficiency. The bacterial consortium was subjected to an application of 100ppm of the mixed dyes for 5 days. Decolorization of the mixed reactive azo dyes (C.I.RY 125) and (C.I.RB 52) and its intermediates were confirmed by FT-IR analysis. The bacterial strains present in the consortium were biochemically characterized and identified by 16s rRNA sequencing. Such alkaliphilic bacterial consortia can be applied in the removal of reactive azo dyes from contaminated alkaline environment.

Keywords: Reactive azo dyes, C.I.RY 125, C.I.RB 52, Bacterial consortium, Textile effluent, Decolorization, Alkaliphilic.

INTRODUCTION

Rapid industrialization has necessitated the manufacture and use of different chemicals in day-to-day life. The textile industry is one of them, which extensively uses synthetic chemicals in the form of dyes (Sathya et al., 2007). Textile manufacturing industries are the backbone of economies in many developed as well as developing countries. In India, it contributes to about 25% of total export earnings and providing employment to almost ¼ of the total labor force (Juwarkar et al., 1997). Pollution due to textile industry effluent has increased during recent



A B

Figure 1. Shows the Structure of the reactive azo dyes (A) C.I. Reactive Yellow 125, (B) C.I. Reactive Blue 52

years. Moreover, it is very difficult to treat textile industry effluents, because of their high Biological Oxidation Demand (BOD), Chemical Oxygen Demand (COD), heat, color, pH and the presence of metal ions (Anjali et al., 2007). Reactive dyes are becoming more popular in their application in textile industry (Zdlinger, 1987). Reactive dyes are chemical compounds capable of forming covalent bond between the dye molecules and the fibers, which are usually cellulose fibers. However, reactive dyes hydrolyze easily resulting in a high portion of unfixed reactive dyes, which have to be washed off during the dyeing process (Shore, 1995). Discharge of effluents without adequate removal of these dyes will remain in the environment and cause serious health and environmental issue (Robinson et al., 2001). This gives rise to lethal effects, geno-toxicity, mutagenicity and carcinogenicity in plants and animals. The disposal of untreated textile water is also one of the major sources of metal dyes, phenols, aromatic amines and these chemicals enter the food chain thereby affecting internal organs such as kidney, liver and the gastrointestinal tract (Racimo et al., 2015).

Several physico-chemical techniques have been proposed for the treatment of colored textile effluents (Ponraj et al., 2011). These are adsorbed on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride, ozone treatment, photo-degradation or membrane filtration (Robinson et al., 2001). These techniques are expensive and produce large amount of secondary pollution (Kalyanasundaram et al., 2001). These cannot be degraded by conventional treatment techniques due to the presence of N=N (azo group) bound to aromatic groups. Hence, economic and safe removal of the polluting dyes is still an important issue. Bioremediation through microorganisms has been identified as a cost effective and environment friendly alternative to these techniques. Mesophilic organisms have limited use in harsh environments, so extremophiles (alkaliphiles and halophiles) being metabolically diverse and tolerant to significant amount of toxic metal are being used in recent studies. The new closed-loop technologies such as reuse of microbial or enzymatic treatment helps in reducing water pollution. Dyeing and printing wastewater has alkaline pH and contains high amount of inorganic salts, which usually inhibit the activities of most microorganisms. For example, the alkali-thermophilic bacteria are also salt tolerant and achieves 90% color removal at pH of 9 and 55◦C, in the presence of high salt content (Alain et al., 2002). Hence it is important to develop a system that can treat the dye containing wastewater by using consortium of alkaliphilic microorganisms (Shah et al., 2014).

The present study focuses on the isolation of alkaliphilic bacterial consortium from textile effluent contaminated site, which could remove mixed azo dyes. The isolated bacterial consortium is used for the decolorization of mixed azo dyes (C.I. Reactive Yellow 125 and C.I. Reactive Blue 52) at different concentrations. The metabolites produced during the degradation of the mixed dyes where identified by FT-IR and GC-MS analysis. Further, the bacterial strains present in the consortium were biochemically characterized and identified by 16s rRNA sequencing. Further, the study focuses on the optimization of growth conditions for alkaliphilic bacterial consortium such as pH, temperature, carbon and nitrogen sources to treat textile effluent wastewater. A bench scale bioreactor test was also done in order to treat synthetic wastewater effluents by the same consortium under the influence of pH, temperature, carbon and nitrogen sources. The resulting decrease in COD levels in the wastewater was analyzed.

MATERIALS AND METHODS

Dyes and chemicals

The textile dyes (C.I Reactive Yellow (C.I.RY 125) 125 and C.I Reactive Blue (C.I.RB 52) 52) were purchased from a textile industry. Figure 1 shows the structure of Reactive azo dyes in this study. All other chemicals used in mineral salts medium and nutrient Agar medium preparation were of analytical grade and purchased from Merck, India.

Sample collection

The bacterial consortium was isolated from soil samples of textile effluent contaminated sites from PTIETC - Pallavaram Tanners Industrial Effluent Treatment Company LTD and C Kalyanam and Co., Pallavaram. The bacterial consortium was enriched in Mineral Salts Medium acclimatized with 100 ppm concentration of each of C.I.RY 125 and C.I.RB 52 Reactive dyes.

Acclimatization of the bacterial consortium and culture conditions

The bacterial consortium was enriched in MSM amended with 100mg/L of C.I.RY 125 and C.I.RB 52. The composition of the MSM used for enrichment and decolorization was as follows: disodium hydrogen phosphate (Na_2HPO_4) 12.8g/L, potassium dihydrogen phosphate (KH_2PO_4) 3g/L, ammonium chloride (NH_4Cl) 1g/L, sodium chloride (NaCl) 0.5g/L, 0.05M magnesium sulphate ($MgSO_4$) 10ml/L, 0.01M calcium chloride ($CaCl_2$) 10ml/L, glucose 20% and pH 9 (Kalyanasundaram et al., 2001). The medium was autoclaved, cooled, and then amended with 100ppm of filter sterilized C.I.RY 125 and C.I.RB 52 in a 250mL Erlenmeyer flask. 10g of soil sample was aseptically inoculated into the MSM. Individual bacterial isolates were obtained from the enriched culture by plating on nutrient agar medium containing 100ppm of C.I.RY 125 and C.I.RB 52. The selected isolates were then purified by streaking on nutrient agar added with 100ppm of the dyes. The single individual pure cultures of the bacterial strains were stored in 15% glycerol at 20˚C for further analysis.

Mixed reactive dyes (C.I.RY 125 and C.I.RB 52) removal at different concentrations

Decolorization of the mixed reactive azo dyes was assayed based on (Alain et al., 2002). To study the decolorization of reactive azo dyes, a volume of 1mL of pre-cultured bacterial consortium was added to 50mL of the MSM and supplemented with different concentrations (100, 200, 300, 400, 500ppm) of C.I.RY 125 and C.I.RB 52. Mineral salts medium added with reactive azo dyes alone served as control (Abiotic control). The decolorization of the mixed reactive dyes (C.I.RY 125 and C.I.RB 52) by the bacterial consortium was observed for every 24h interval for 5days. The decolorization of mixed dyes were monitored by withdrawing the samples periodically and then centrifuged at 10,000g for 15min and decolorization was measured using UV/Vis spectroscopy (Hitachi) at the corresponding λ max of the dye and was compared with the abiotic control. The total protein content was also estimated at every 24-hour interval. All decolorization experiments were carried out in duplicates. The color removal efficiency of the bacterial consortium was determined as follows:

% Decolorization = (A-B)/A × 100;

Where A is the initial absorbance of control dye and **B** is observed absorbance of degraded dye.

Analysis of Metabolites in the decolorization study

FTIR Spectroscopy analysis

Biodegradation of C.I.RY 125 and C.I.RB 52 by bacterial consortium was analyzed by FTIR. For this, 100 ml of culture broth was taken at 0 hr and 72hr (before and after decolorization), centrifuged at 10,000rpm for 15mins and the degraded metabolites were extracted from supernatant using equal volumes of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator (Shah et al., 2014). The samples were mixed with KBr (1:20; 0.02g of sample with KBr at a final weight of 0.4g) and ground, desorbed at 60˚C for 24h and pressed to obtain IR-transparent pellets. The absorbance FT-IR spectra of the samples were recorded using an FTIR Instrument model (IR Affinity-1 (SHIMADZU), Ethiraj College for Women, Chennai. The spectra were recorded within a scanning range of 400-4000 cm^{-1}. The instrument was first calibrated for background signal scanning with a control sample of pure KBr and then the experimental samples were scanned. The FT-IR spectrum of the control was finally subtracted from the spectra of the non-degraded and degraded dyes.

GC-MS analysis of degraded product

The bacterial strain was inoculated in minimal medium containing 100mg/l of textile mixed reactive dyes C.I.RY 125 and C.I.RB 52 and incubated under ambient conditions. After complete decolorization, 40ml of degraded samples was taken and centrifuged at 10,000rpm for 10min. Then the supernatant was filtered through Whatman #1 filter paper. Filtrate was then extracted three times with diethyl ether pooled and evaporated the extracted product to dryness. The degraded products were analyzed using TLC and GC-MS. For TLC, the solvent system butanol: water: acetic acid = 5:3:2 was poured it into the TLC tank. The extracted sample was dissolved in 0.5ml of methanol and was loaded on TLC plate of 3.5 × 5.0cm and allowed to run in a TLC tank. After the run was complete the TLC plate was taken out and air-dried. The plate was observed under UV light and the R_f values of the bands were recorded. The extracted metabolites were also analyzed by the gas chromatography-mass spectrometry

carried out with JEOL GC Mate-II fitted with HP5 column.

Optimization of growth conditions for decolorization of C.I.RY 125 and C.I.RB 52

Effect of pH and Temperature on the decolorization of mixed dyes

In order to study the effect of pH and temperature, the sterilized MSM was amended with 100ppm of each of the (C.I.RY 125 and C.I.RB 52) dyes. The medium was maintained at different pH: 8, 8.5, 9, 9.5, and 10 respectively. A volume of 1ml of overnight culture was inoculated in the flasks and incubated in a shaker at 36˚C. The effect of temperature was studied by inoculating the culture and incubating in a shaker at 28˚C, 37˚C, and 45˚C. The measurement of decolorization of the total dye concentration and total protein estimation was performed at every 24 h interval for 5 days (Sylvine and Veena, 2016).

Effect of Carbon and Nitrogen sources on the decolorization of mixed dyes

The effect of carbon sources was studied using various compounds, such as Maltose (M), Sucrose (S), Lactose (L) and Xylose (X), at a concentration of 1% and they were added individually as a supplement to MSM for the decolorization of mixed reactive azo dyes. A volume of 1ml of the overnight culture was inoculated in the flasks and incubated in a shaker at 37˚C. Nitrogen sources such as ammonium acetate (AA), ammonium nitrate (AN), ammonium oxalate (AO) and yeast extract powder (YEP) were added to MSM at a concentration of 1%; and 1ml of overnight culture was incubated at 36◦C. In order to study the effect of efficient carbon and nitrogen sources, the optimum carbon and nitrogen sources, i.e., maltose (M) and ammonium nitrate (AN), were added to MSM at a concentration of 1%, respectively, and the decolorization of the dyes were measured.

Scanning electron microscopy

The sample preparation for Scanning Electron Microscopy (SEM) was carried out according to the method of Prior and Perkins (Pokharia and Ahluwalia, 2013). The isolated bacterial strains were grown individually on MSM for 24h. The bacterial strains in the mineral salts medium were centrifuged at 8000g for 10min and the pellets were immediately re-suspended in 2% glutaraldehyde with 0.05M phosphate buffer and 4% sucrose (pH 7.3). Fixation was obtained overnight at 4°C. After 24 hours the pellets were centrifuged at 8000g for 10min, washed 4 times with distilled water, placed on aluminum foil. The samples were then dehydrated with series of gradient ethanol (i.e 10%, 20%, 30% till 90%), air dried and finally the dried flakes were coated with platinum and examined under SEM (JEOL JSM-6360).

Identification of reactive azo-dye degrading bacterial strains

The individual bacterial strains were separated from the consortium, and were used for the degradation of the azo dyes. The bacterial strains present in the consortium were initially examined using conventional biochemical tests. The molecular identification of bacterial strains was performed by 16S rRNA sequencing. The bacterial strains present in the consortium were isolated and grown separately. Initially, Gram staining and motility tests were performed and then the biochemical characterization was carried out for different parameters (catalase, oxidase, indole production, citrate utilization, triple sugar, iron, agar, and urease) using 24h old culture of individual bacterial strains. After 24h of incubation at 37◦C, the color change observed was accounted for a positive/negative result. The genus level identification of the unknown bacterial strains was carried out using Bergey's Manual of Systematic Bacteriology (Bauer et al., 1966) to ascertain the existence of variable biochemical test results for each strain.

16S rDNA Partial gene sequencing

Chromosomal DNA was isolated from pure strains of the consortium by the standard phenol/chloroform extraction method (Bauer et al., 1966). The 1.5kb partial sequence of 16S rDNA gene was amplified by the chromosomal DNA using polymerase chain reaction (PCR) with universal Eubacteria-specific primers 16F27 (5-CCA GAG TTT GAT CMT GGC TCA G-3) and 16R1525XP (5-TTCTGCAGT CTA GAA GGA GGT GWT CCAGCC-3). The used PCR conditions were: initial denaturation at 94◦C for 2min, followed by 35 cycles of denaturation at 95◦C for 1min, annealing at 55◦C for 1min, extension at 72◦C for 1min, and a final extension at 72◦C for 10min; and finally sequencing was performed on an ABI310-automated DNA sequencer using Big Dye terminator kit (Applied Biosystems 3730x l DNA Analyzer). The amplified 16S rDNA gene (PCR products) from these isolates was directly sequenced after purification by precipitation with polyethylene glycol and NaCl. The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as used for the PCR amplification (16F27N and 16R1525XP).

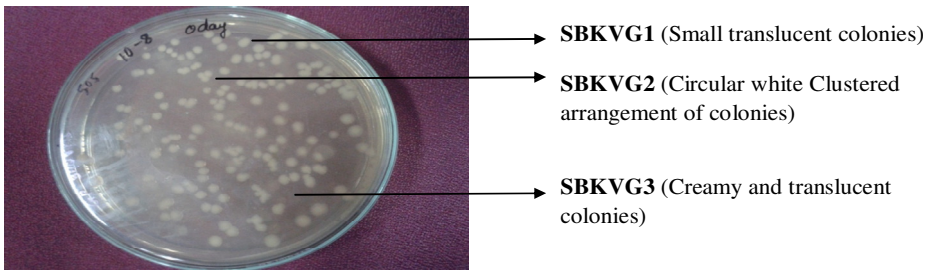


Figure 2. Enriched bacterial isolates on MSM containing C.I.RY 125 and C.I.RB 52 dyes (100ppm).

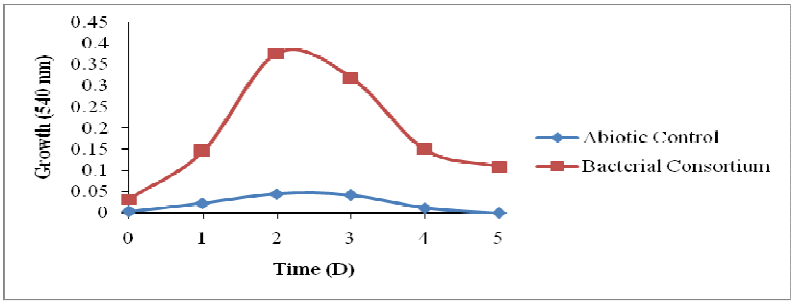


Figure 3. Growth of the Bacterial consortium during enrichment period

Phylogenetic analysis

The output file of sequence alignment was used to compute phylogenetic trees for aligned sequences of 16s r-RNA sequencing results of the four bacterial strains (SBKVG1, SBKVG2 and SBKVG3). Neighbor joining method was used for tree building with MEGA 6 software. To access the reliability of the phylogenetic tree, MEGA provides bootstrap test which used the bootstrap resampling strategy. The user has to input the number of replicates. In this experiment, 500 replicates were used.

Bench scale bioreactor studies with synthetic textile effluent wastewater

The isolated bacterial consortium which was able to degrade mixed reactive azo dyes (C.I.RY 125 and C.I.RB 52) under alkaline conditions was used in the treatment of textile effluent containing contaminated wastewater in a lab-scale bioreactor. The preliminary study was conducted on the treatment of synthetic textile effluent contaminated wastewater prior to laboratory scale bioreactor study. In the preliminary experiments, batch study was performed in shake flasks with combined synthetic textile effluent contaminated wastewater supplemented with mineral medium (1:1 v/v) along with the bacterial consortium. De-colorization of mixed reactive azo dyes (C.I.RY 125 and C.I.RB 52) in the treatment of textile effluent containing contaminated wastewater and total protein content were estimated. The effect of pH, carbon and nitrogen sources was estimated on the total COD removal efficiency of the consortium.

RESULTS AND DISCUSSION

Screening and isolation of bacterial consortium degrading the mixed reactive azo dyes (C.I.RY 125 and C.I.RB 52)

The initial enrichment of the bacterial consortium for the azo-dye degradation indicated four bacterial strains out of which only three bacterial strains were able to decolorize C.I.RY 125 and C.I.RB 52 (100ppm) under alkaline conditions. The bacterial strains present in the consortium were labeled as SBKVG1, SBKVG2 and SBKVG3, which utilized the reactive dyes as a sole carbon and energy source. All the three bacterial isolates were further grown on MSM-containing agar without addition of any carbon and nitrogen sources. They showed the ability to grow on the MSM agar after 48 h of incubation at 37˚C. The screening experiments for dye removal were carried out under alkaline pH. The isolated bacterial consortium enriched in MSM was amended with reactive dyes on nutrient agar containing the reactive dye is shown in Figure 2. Figure 3 shows that there was an increase in growth of the bacterial consortium from 1st day (0.145) to 2nd day (0.376). By the end of the 3rd day (0.319), there was decrease in growth and bacterial

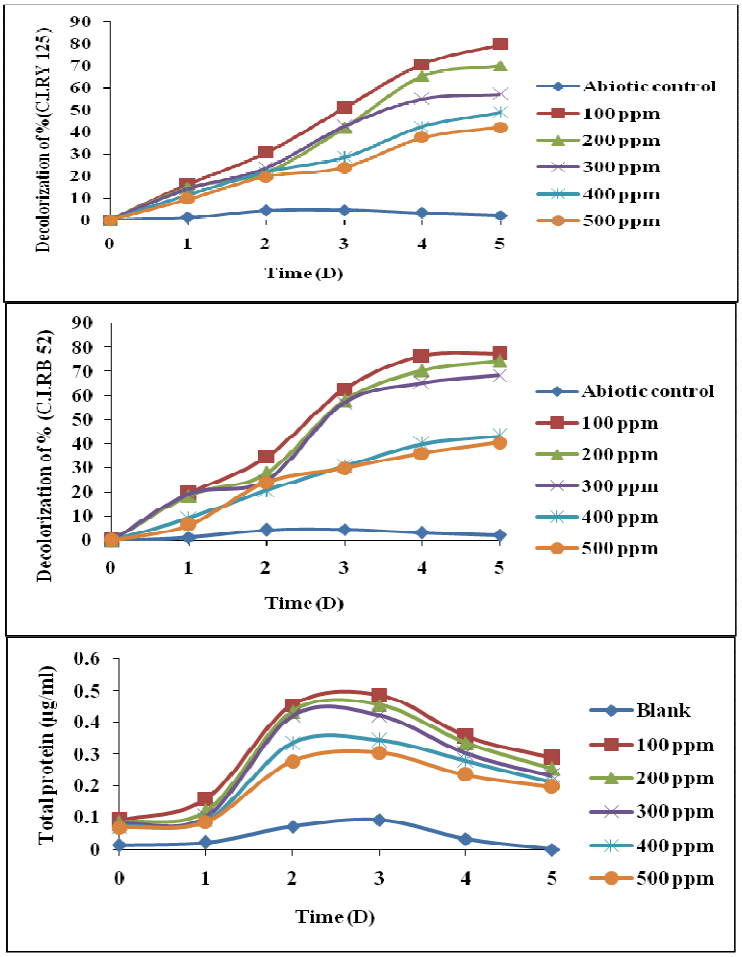


Figure 4. Decolorization of various concentration of mixed reactive dyes (C.I.RY 125 and C.I.RB 52) and total protein content

consortium could not survive after the 5th day of the growth experiment. Hence for all the further experiments, the growth and decolorization of mixed dyes was checked up to 5th day of incubation.

Mixed reactive azo dyes (C.I.RY 125 and C.I.RB 52) decolorization at different concentrations

The ability of the isolated bacterial consortium to use C.I.RY 125 and C.I.RB 52 dyes as a sole carbon and energy source was studied at 100ppm to 500ppm (100ppm, 200ppm, 300ppm, 400ppm and 500ppm). The Reactive dye (C.I.RY 125) decolorization at 100ppm concentration varied from 16.3% (1st day) to 79.6% at the end of the 5th day. When the concentration of C.I.RY 125 dye was increased to 200ppm, 300ppm, 400ppm and 500ppm the decolorization efficiency started to decrease to 70.2%, 57.2%, 48.9% and 42.1% at the end of the 5th day. C.I.RB 52 decolorization at 100 ppm concentration varied from 19.4% (1st day) to 77.7% at the end of the 5th day. When the concentration of C.I.RY 125 dye was increased to 200ppm, 300ppm, 400ppm and 500ppm the decolorization efficiency started to decrease to 74.6%, 68.6%, 43.4% and 40.6% at the end of the 5th day. At a concentration of 100 ppm the total protein content of the isolated bacterial consortium was maximum at the third day (66µg/ml). The maximum initial decolorization of C.I.RY 125 and C.I.RB 52 dyes by the bacterial consortium was observed at the concentration of 100 ppm and as well as the maximum total protein content of bacterial consortium was also found to be at the same concentration. The increase in the total protein content and removal of C.I.RY 125 and C.I.RB 52 dyes at various concentrations is mentioned in the Figure 4. These study concords with an earlier study of *Bacillus subtilis, Pseudomonas aeruginosa, Pseudomonas fluorescens, Serratia marcescens* and *Escherichia coli* extracted from textile dye effluents to evaluate their potential to decolorize Reactive Red M5B, Reactive Blue 19, Reactive Yellow 44, Reactive Orange M2R and Reactive Green 19 (Deininger, 1990).

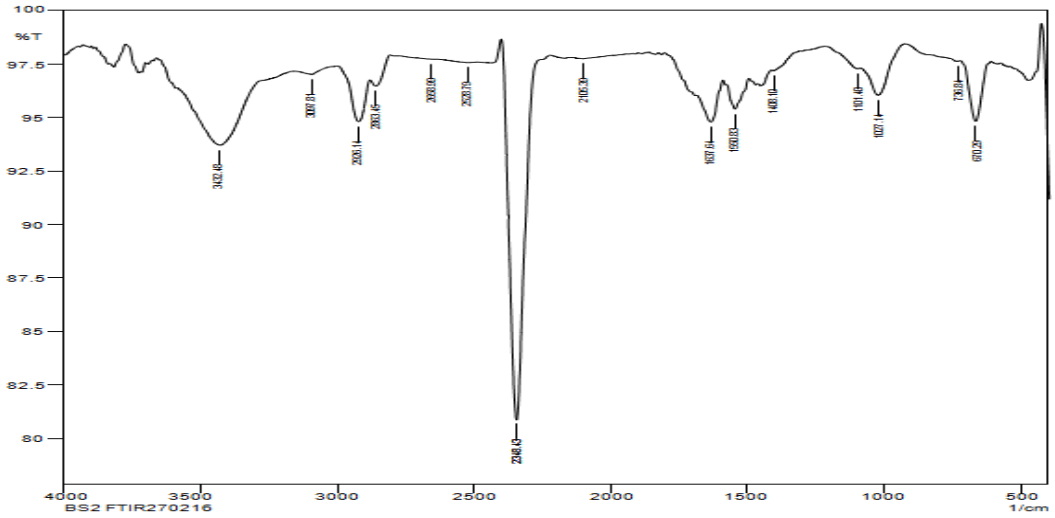


Figure 5. FTIR Spectrum of Mixed Reactive Azo dyes (C.I.RY 125 and C.I.RB 52)

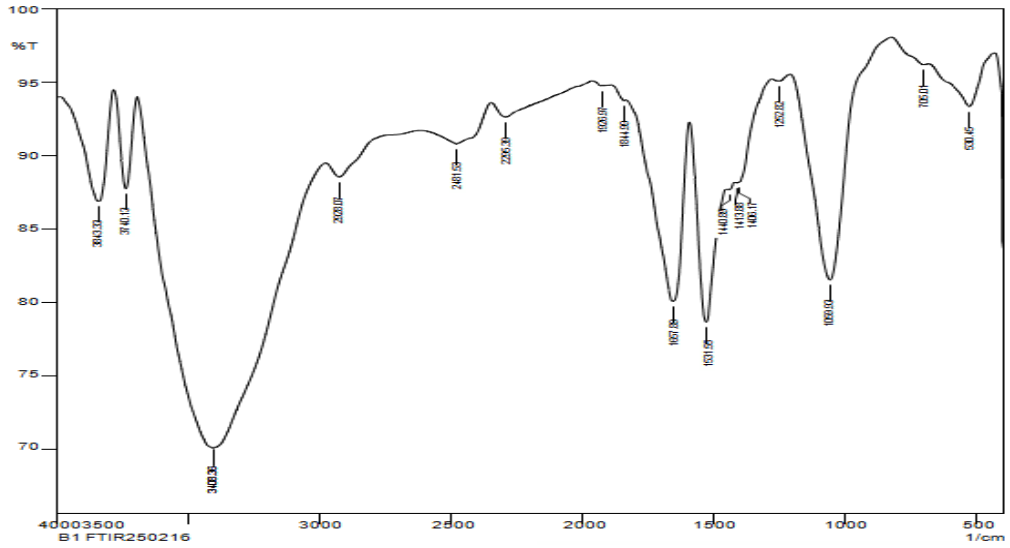


Figure 6. FTIR Spectrum of degraded metabolite by the bacterial consortium (C.I.RY 125 and C.I.RB 52)

Analysis of metabolites during the decolorization study

FTIR Analysis

The FTIR spectrum of the mixed azo reactive dyes (C.I.RY 125 and C.I RB 52) used as a control and that of decolorized mixed reactive azo dyes by isolated bacterial consortium (72hours) were compared to evaluate the production of metabolites during the decolorization. The spectrum of the control dye (Figure 5) displayed a peak at 3408.36 cm^{-1} indicating the presence of N-H stretching. The 1657.89cm^{-1} peak indicates the presence of (-N=N-) azo group. The peak at 1531.55cm^{-1} is present due to N-H, C=C, C=N as plane bending (primary amine). The peak at 1252.82cm^{-1} represented S=O stretching frequency of SO_3Na group of aromatic ring. The peak at 1059.93cm^{-1} represented the presence of C-F halogen.

After decolorization, the FTIR Spectrum of extracted metabolites (72h) by the isolated bacterial consortium (Figure 6) showed a considerable change in the positions of peaks as compared to the spectrum of control dye. A new peak at 3432.48cm^{-1} and 2926.14cm^{-1} was observed due to the presence of N-H stretching and C-H stretching in the extracted metabolite. The largest narrow peak at 2348.43cm^{-1} represented the carbon di-oxide asymmetric stretching, which is a miscellaneous compound produced during the decolorization of mixed reactive dyes. The

peak at 670.29cm^{-1} indicates the C-S stretching in the degraded products. The reduction of peaks at 1637.64cm^{-1} indicates the reductive cleavage of C.I.RY 125 and C.I.RB 52 dyes at the azo bond (-N=N-) when compared to control dye spectrum. The peak at 1550.83cm^{-1} is found due to the degradation of primary amine N-H, C=N and C=C plane bending of aromatic rings respectively. The reduction peak at 1027.14 cm^{-1} indicates the degradation of C-F halogen. The three peak values (1637.64cm^{-1}, 1550.83cm^{-1}, 1027.14cm^{-1}) which are highlighted in the FTIR spectrum proves that the changes in functional groups of the C.I.RY 125 and C.I.RB 52 reactive dyes. The breakage of the aromatic ring of the mixed reactive azo dye and formation of amino groups clearly indicated the decolorization of mixed reactive azo dyes by the bacterial consortium under alkaliphilic conditions. Earlier studies reported the FTIR spectrum of extracted products after decolorization of dye RB 172 showing variation in the positions of peaks when compared to control dye spectrum. The disappearance of peaks at 1620.29 cm^{-1} and 1598.51cm^{-1} indicates the reductive cleavage of dye RB 172 at azo bond position. The peak at 2974.86cm^{-1} indicates the C–H stretching of alkanes while the peak at 2833.71cm^{-1} shows C–H stretching of ethers. The peak obtained at 1442.05cm^{-1} is due to C–H deformation of alkanes. In addition, peak at 1246.96cm^{-1} shows O–NO_2 vibration of nitrates while the peak at 1067.06 cm^{-1} suggests C–OH stretching of primary alcohols. These changes in the FTIR spectrum are clear evidence for the degradation of dye RB 172 into simpler molecules, like aliphatic amines and carboxylic acids (Dos Santos et al., 2006).

GC-MS Analysis

The mass spectral analysis was performed for checking the intermediates produced during the decolorization of mixed dyes (C.I.RY 125 and C.I R.B 52). The spectrum analysis showed the degradation of the mixed dyes with the production of intermediates which was revealed by the presence of several peak values which were obtained on the basis fragmentation pattern at m/z values. The GC-MS analysis produced several peaks showing the presence of intermediate metabolites produced during the degradation of the mixed dye. The chromatogram and spectra (Figure 7 below) shows two prominent peaks with RT (retention times) of 10.27 and 27.10. Mass spectra corresponding to the peak with RT value of 10.27 indicates a fragmentation pattern with m/z values of 44, 73, 96, 133, 135, 147, 191, 207, 208, 209, 281, which indicates the presence of hexamethyl cyclotrisiloxane, an aromatic compound which is degraded by the bacterial consortium. Hexamethyl cyclotrisiloxane is an aromatic amine and the presence of it is a cardinal sign of degradation of the mixed dyes by the bacterial consortium. In addition, there are several other metabolites such as silicic acid, diethyl-bis-(trimethylsilyl) ester. Two important intermediate compounds were reported from the analysis of the degraded mixed dyes. One of the intermediate was found to be benzyl alcohol which showed peak values at 44, 78, 108, 207m/z.

RT value of 10.27 may be due to the presence of cyclotrisiloxane. A small peak with the RT value of 27.10 gave mass spectra with m/z 44, 78, 108, 207 due to the presence of benzyl alcohol. The bacterial degradation of azo dyes was reported to start with the reduction of azo bond which is generally a non-specific and presumably extracellular process in which the reducing equivalents from an external electron donor (biologically or chemically generated) are transferred to the dye resulting in the production of aromatic amines. This reduction usually takes place in the absence of oxygen and many microorganisms are known to possess an enzyme azoreductase that may be oxygen insensitive (Rajaganesh and Ameer, 2014; Lade et al., 2015; Prasad and Rao, 2013; Pearce et al., 2003; Zimmermann et al., 1982). These aromatic amines require the presence of oxygen for further breakdown as these oxygen molecules have to be inserted into such molecules which initiate other metabolic degradation (Mazumder et al., 1999). The presence of salycilic acid confirmed by GC-MS clearly indicates that the naphthalene part of the dye must have undergone oxidative degradation followed by decarboxylation to hexamethyl cyclotrisiloxane. Since the degradation was performed to analyze two complex aromatic ring structures, during the degradation process, the consortium might have almost degraded one of the dyes to hexamethyl cyclotrisiloxane and the other to benzyl alcohol. Further individual dye degradation study with the isolated consortium and the prediction of enzymes involved in the degradation process will provide us a clear picture of the compound which has undergone complete degradation. The final goal of this work is to study the effect of alkalophilic bacteria on the degradation of mixed dyes which on par to the raw textile effluent waste water.

Optimization of growth conditions

Effect of pH on the decolorization of dye mixture (100ppm) and experimental validation

Alkaline pH plays an important role in the metabolism of alkaliphilic bacteria. The study was carried out to determine the effect of pH (pH 8, pH 8.5, pH 9, pH9.5, pH 10) on the decolorization of C.I.RY 125 and C.I.RB 52 dyes at optimum concentration of 100 ppm respectively. The study on the effect of pH on the decolourization of the mixed reactive azo dyes proved that the bacterial consortium was able to grow at an optimum pH of 9 in

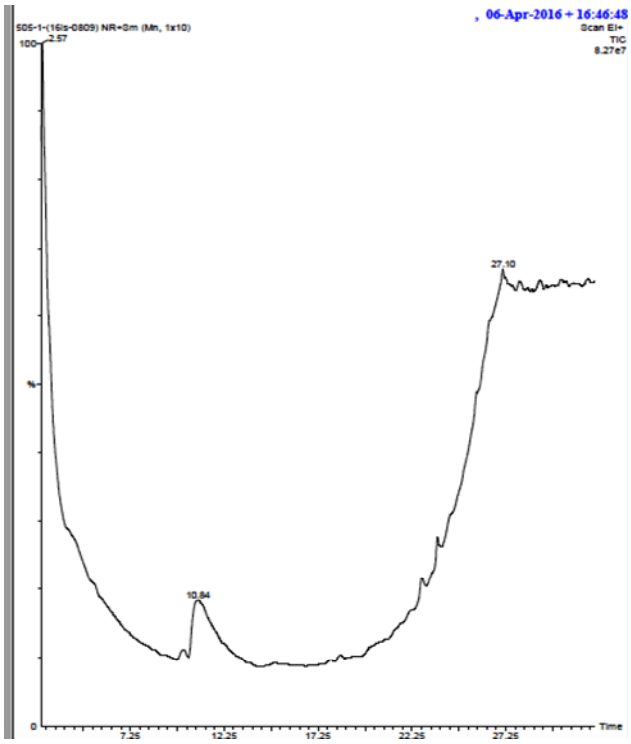


Figure7. GC-MS Peaks with Retention Time (RT) for degraded metabolite by the bacterial consortium

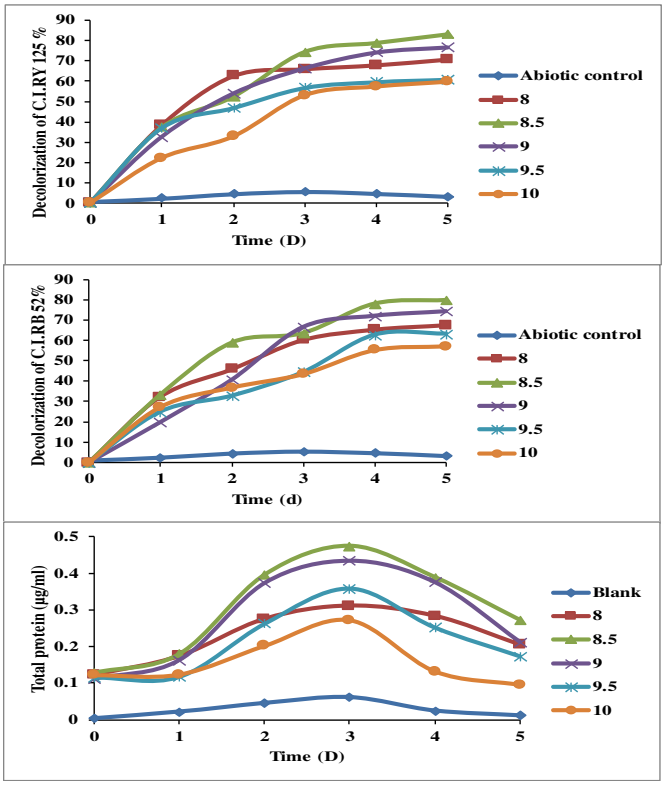


Figure 8. Effect of pH on decolorization of Mixed reactive azo dyes C.I.RY 125 and C.I.RB 52 Dye and total protein content (100 ppm)

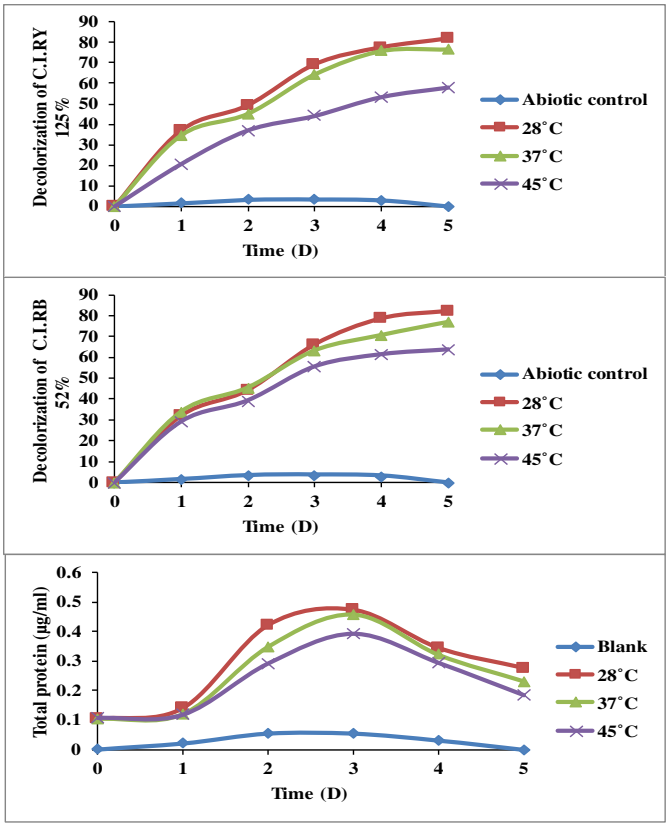


Figure 9. Decolorization of mixed Reactive azo dyes (C.I.RY 125 and C.I.RB 52) at different temperature by the isolated bacterial consortium

MSM which shows isolated bacterial consortium was alkaliphilic in nature. In C.I.RY 125 and C.I.RB52 dyes the maximum decolorization was observed at pH 8.5 which was found to be 82.9% and 79.8% at the end of 5th day. Minimum decolorization of C.I.RY 125 and C.I.RB52 dyes was found to be 59.5% and 57.1% at pH 10 respectively. The total protein content was found to be maximum at pH 8.5 (50µg/ml). The increase in the total protein content and removal of C.I.RY 125 and C.I.RB 52 dyes at various pH is represented in the Figure 8.

Similar studies reported on the effect of pH (7, 8, and 9) on decolorization of four reactive dyes (reactive red, reactive yellow, reactive brilliant red and reactive brilliant blue) at 50mg/l concentration of dye with 20% inoculums that the percentage removal of dye increased with increase in incubation period at pH 9. The maximum removal of ETL-1949 were 80%, 73%, 63% and 63.5% respectively found at pH 9 by Bacillus spp., after 7 days of incubation. At pH 7 and 8, percentage removal of dye decreased when compared to pH 9; the optimum pH was found to be 9 for maximum removal of dye (Shah et al., 2014). The pH tolerance of decolorizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and at high temperature. Other studies revealed that the increase in pH from 4 to 8 significantly enhanced the decolorization of Reactive Black dye and increase in pH beyond 8 decreased the decolorization capacity by all selected bacterial strains (ETL-1, ETL-2, ETL-12, ETL-14, ETL-16) (Shah et al., 2013). In another report, it was found that Halomonas sp. was reported to decolorize up to 90% of azo dyes in the presence of NaCl in narrow pH range of 6.5-8.5 (Asad et al., 2014).

Effect of temperature on the decolorization of dye mixture (100ppm) and experimental validation

Effect of temperature (28˚C, 37˚C, and 45˚C) were studied by inoculating the isolated bacterial consortium in MSM containing C.I.RY 125 and C.I.RB 52 dyes at an optimum concentration of 100ppm respectively. At 28˚C, enhanced bacterial consortium showed maximum decolorization of the mixed dyes which was found to be 81.7% (C.I.RY 125) and 82.3% (C.I.RB 52) by the end of 5th day. At 37˚C showed the maximum decolorization of the mixed reactive dyes which was found to be 76.5% (C.I.RY 125) and 77.1% (C.I.RB 52). AT 45˚C showed the least decolourization of Reactive azo dyes was observed which was found to be 58% in C.I.RY 125 dye and 63.8% in C.I.RB 52 dye. Total protein content was found to be maximum at 28˚C (50µg/ml). This experiment

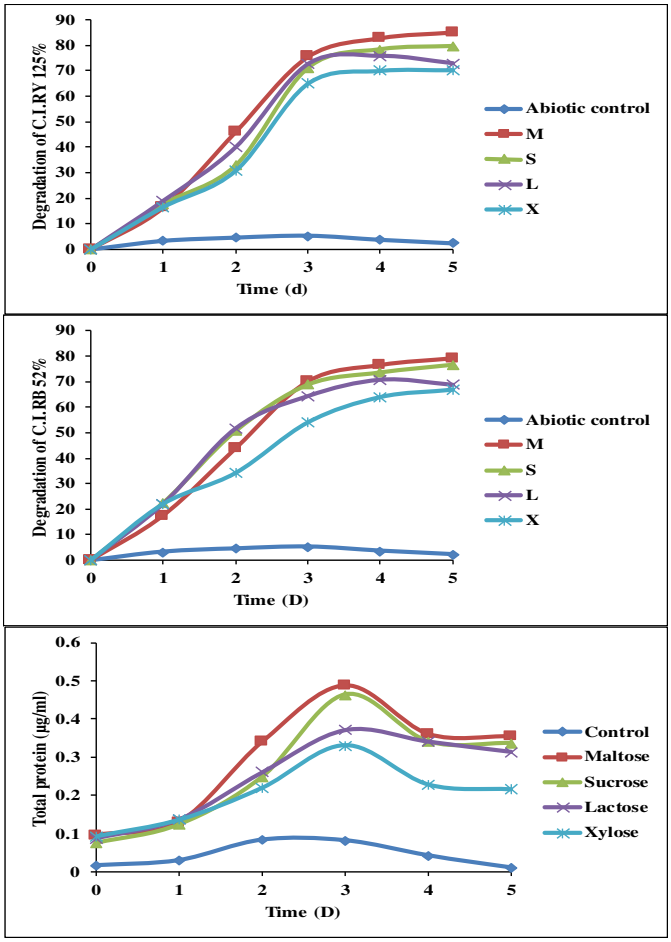


Figure 10. Effect of carbon sources on decolorization of mixed reactive dyes (C.I.RY 125 and C.I.RB 52) and total protein content (100 ppm).

showed that the bacterial consortium could survive at various temperatures but showed optimum decolorization at 28˚C. The increase in the total protein content and removal of C.I.RY 125 and C.I.RB 52 dyes at various temperatures is mentioned in the Figure 9.

It has been reported that the optimum temperature of decolorization of Reactive red 31 (55.67%) and Reactive yellow 81 (59.89%) to be 35ºC (Pradhan and Kumar, 2012). Also, it was found that there were 16 isolates out of which 2 isolates (Enterococcus sp. and Klebsiella sp.) were found to be able to decolorize the Reactive Blue 19 at different temperatures ranging from 30 to 100˚C. Enterococcus sp. and Klebsiella sp. gave maximum decolorization at 37˚C after 72 hours of incubation (Gulati and Jha, 2014). On increasing the temperature from 35◦C to 40◦C, there is a sharp decrease in the decolorization in all isolates. Decline in the decolorization activity at higher temperature can be attributed to loss of cell viability.

Effect of carbon sources on the decolorization of dye mixture (100ppm) and experimental validation

To study the influence of carbon sources on the decolorization of mixed dyes at optimum concentration (100ppm), carbon sources like Maltose (M), Sucrose (S), Lactose (L), Xylose (X) were supplemented in the media along with C.I.RY 125 and C.I.RB 52 dyes. Figure 10 shows that almost all the carbon sources were able to enhance the decolorization of C.I.RY 125 and C.I.RB 52 dyes. An increase in decolorization was observed in the batch study when maltose was used as the carbon source; it showed maximum decolourization of C.I.RY 125 (85.2%) and C.I.RB 52 dyes (79.3%) by the end of 5th day. This was followed by Sucrose, Lactose and Xylose which showed decolorization of C.I.RY 125 dye up to 79.7%, 72.9%, 70.3% and C.I.RB 52 up to 76.7%, 68.9%, 66.9% respectively, by the end of the 5th day. Total protein content was found to be maximum (64µg/ml) with Maltose at the 3rd day. Figure 7 above shows the graphical representation of decolorization of

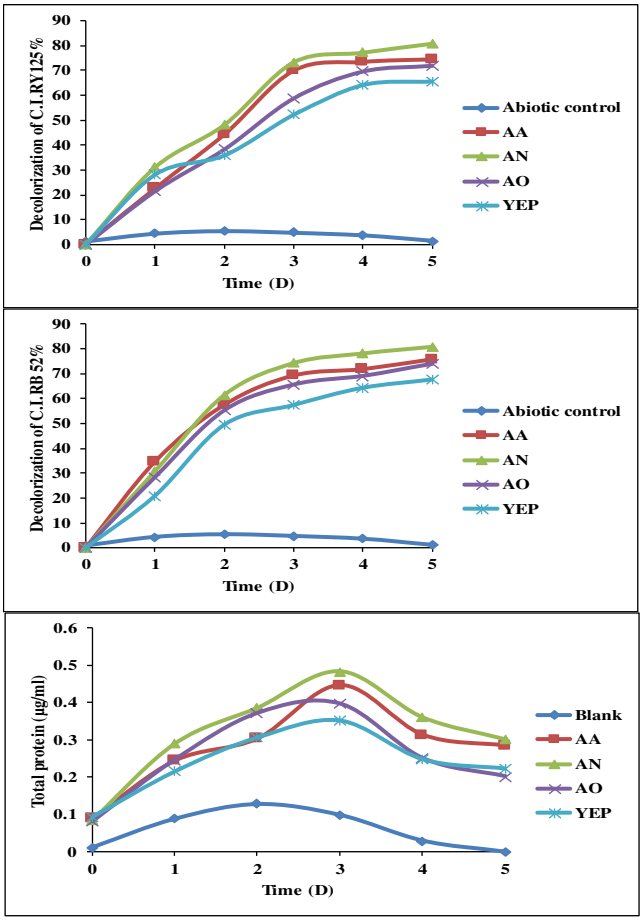


Figure 11. Effect of nitrogen sources on decolorization of Mixed reactive azo dyes (C.I.RY 125 and C.I.RB 52) and total protein content and (100ppm)

C.I.RY 125 and C.I.RB 52 dyes by bacterial consortium and maximum total protein content with Maltose as the sole carbon source.

A study on the effect of different carbon sources (xylose, glucose, sucrose, maltose and starch) on decolorization of Reactive Red 195 and Reactive Orange 72 by P. putida and Reactive Yellow 17 and Reactive Blue 36 by B. licheniformis has been done. Glucose has emerged as the ideal carbon source for both bacteria, all recording highest rate of decolorization (85.07 and 71.90, 82.21 and 74.82% respectively), but xylose recorded least percentage decolorization by both bacteria (below 48%) (Suganya et al., 2014).

Effect of nitrogen sources on the decolorization of dye mixture (100ppm) and experimental validation

To study the influence of nitrogen sources on the decolorization of C.I.RY 125 and C.I.RB 52 dyes at optimum concentration (100ppm), various nitrogen sources like Ammonium acetate (AA), Ammonium nitrate (AN), Ammonium oxalate (AO) and Yeast extract (YE) were supplemented in MSM along with C.I.RY 125 and C.I.RB 52 dyes. Figure 11 shows that the addition of nitrogen sources could enhance the growth and decolorization of mixed dyes. Ammonium nitrate showed the maximum decolorization of C.I.RY 125 and C.I.RB 52 dyes, which was found to be 80.8 % and 81.0% by the end of 5th day respectively. This was followed by Ammonium acetate, Ammonium oxalate and Yeast extract with C.I.RY 125 giving decolorization percentages upto 74.5%, 72.0%, 65.7% and C.I.RB 52 upto 75.8%, 74.1%, 67.9% respectively by the end of 5th day. Total protein content was found to be maximum (61µg/ml) on the 4th day in the media supplemented with ammonium nitrate. Yeast extract supplemented media showed the least decolourization - 65.7% and 67.9% and it did not play effective role in decolorization of C.I.RY 125 and C.I.RB 52 dyes. Figure 12 shows the maximum decolorization of C.I.RY 125 and C.I.RB 52 dyes by bacterial consortium and maximum total protein content with ammonium nitrate as nitrogen source.

It was proved that Lysinibacillus sphaericus strain RSV-

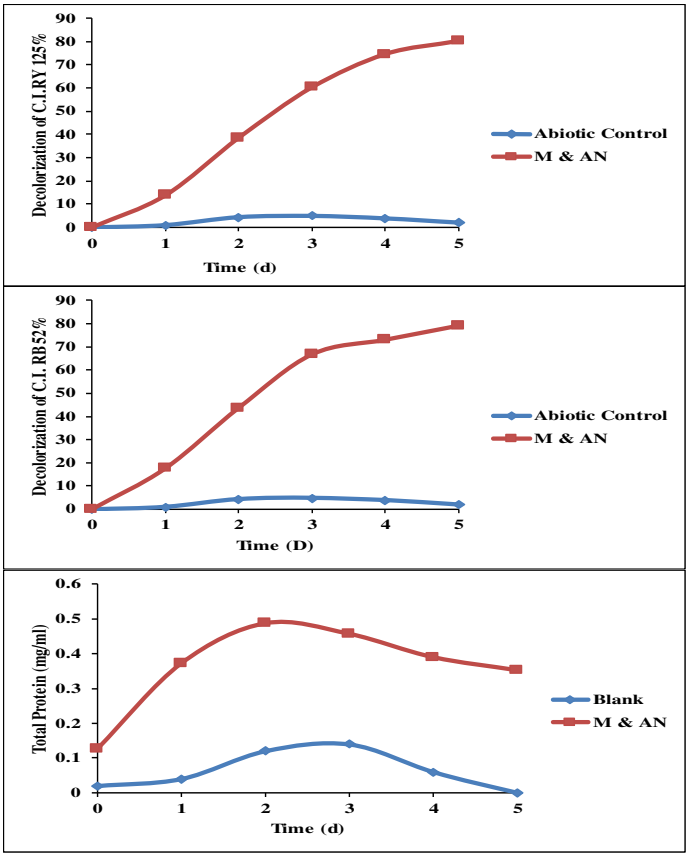
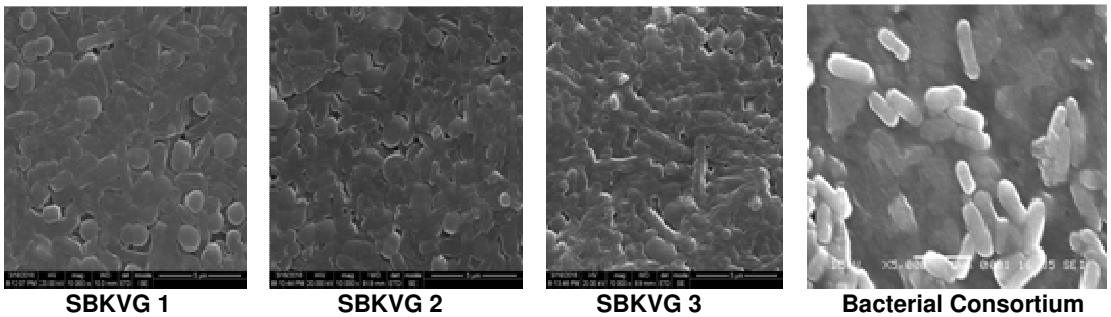


Figure 12. Effect of Mixed carbon and nitrogen sources on decolorization of mixed reactive azo dyes (C.I.RY 125 and C.I.RB 52) and total protein content



SBKVG 1 (Coccoid bacilli Shape) **SBKVG 2 (Rod Shaped Bacilli)** **SBKVG 3 (Rod shaped Bacilli)** **Bacterial Consortium**

Figures 13. Morphology of the individual bacterial strains and consortium under 11000 X magnification.

1 couldn't grow and decolorize the dyes (Yellow ME4GL, Blue RR, Red RR, Yellow RR, Red M5B, Blue MR, Deep Black RR, Yellow MERL, Red ME4BL and Golden Yellow (1000ppm) incorporated in inorganic nitrogen supplements such as ammonium nitrate, urea, ammonium chloride and ammonium sulphate. But the strain effectively utilized the dyes in the presence of yeast extract and decolorized the dyes up to 95% within 2hrs (Rajeswari et al., 2014). Also, it was proved that the addition of inorganic nutrients is not always effective to stimulate the degradation because many factors can decrease the biodegradation and addition of inorganic nutrients change the pH.

Effect of carbon and nitrogen sources on the decolorization of dye mixture (100ppm) and experimental validation

To increase the degradation efficiency, the Carbon and

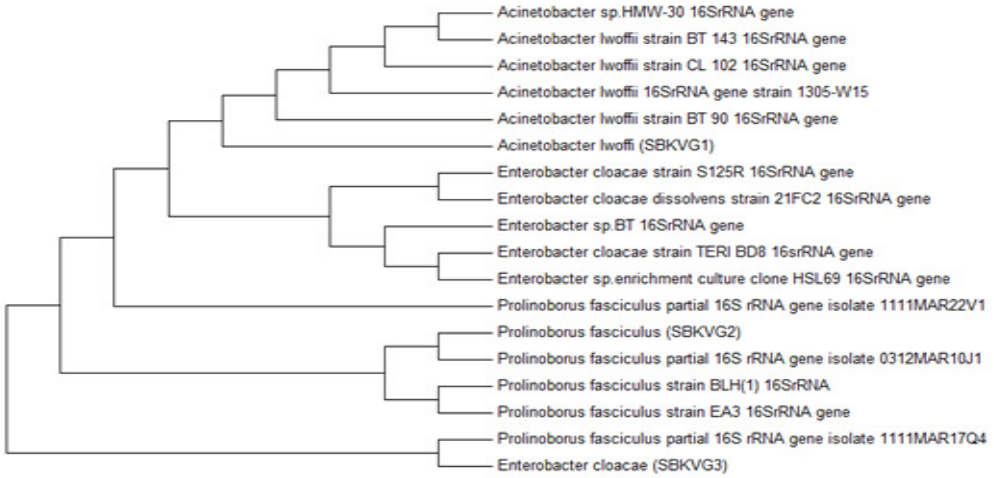


Figure 14. Phylogenetic analysis of *Acinetobacter lwoffii, Prolinoborus fasciculus and Enterobacter cloacae subsp*. strains with its nearby sub strains.

Nitrogen sources which showed the maximum decolorization were combined and dye degradation was checked at optimum concentration (100ppm). In this experiment, Maltose (M), which served as optimum carbon source and ammonium nitrate (AN), which served as optimum Nitrogen source (1% and 1%) were supplemented in MSM. Figure 12 shows that the addition of a combination of Maltose and Ammonium nitrate was the
ideal carbon and nitrogen source for decolorization of C.I.RY 125 and C.I.RB 52 dyes by the isolated bacterial consortium. Maltose (M) and Ammonium nitrate (AN) could further enhance the decolorization of C.I.RY 125 and C.I.RB 52 dyes, which was found to be 80.3% and 76.2% by the end of 5th day respectively. Maximum total protein content was found to be (67µg/ml) at the end of 2nd day. It shows the decolorization of mixed dyes by bacterial consortium and the maximum total protein content observed using combination of maltose and ammonium nitrate as carbon and nitrogen sources.

It has been reported that the dye degradation efficiency increased when sucrose and yeast extract (1% and 0.5%) were added to the media as the carbon and energy source to decolorize the azo dyes (Direct Blue 151 and Direct Red 31) by alkaliphilic bacterial consortium (B.cereus, B.cyto-toxicus, Bacillus sp. L10, and B.flexus). The addition of a combination of yeast extract and sucrose further enhanced the decolorization of mixed dyes by 94% at the end of the 5th day (Sylvine and Veena, 2016).

SEM analysis of bacterial strains

SEM analysis was carried out to determine the morphological structures of both the individual bacterial as well as the bacterial consortium. The ultra structure of the bacterial strains was observed under 10,000x magnification (FEIN QUANTA-200F, IIT, Chennai). Figure 13 shows the structure of the bacterial strain SBKVG1, SBKVG2, SBKVG3 and the bacterial consortium respectively under 10,000x magnification. It was seen that the individual bacterial strains SBKVG1, SBKVG2, SBKVG3 and the consortium showed coccoid bacilli and rod shaped bacilli morphology.

Genomic DNA extraction and PCR amplification

The genomic DNA was isolated from each bacterial isolates by the standard Phenol/Chloroform extraction method. PCR amplification was performed and the unknown bacterial strains were identified through 16S r-DNA sequencing. The bacterial isolates were identified from the sequence using BLAST tool.

Identification of the isolated bacterial consortium by phylogenetic analysis

The Bacterial strains (SBKVG1, SBKVG2 and SBKVG3) belong to the phylum Proteobacteria. The phylogenetic tree (Figure 14) constructed by MEGA-6 displayed a relation between all the isolated bacterial strains. Phylogenetic analysis based on nucleotide sequences from SBKVG1 showed a maximum of 99% identity towards *Acinetobacter lwoffii* strain BT90. Hence the SBKVG1 was identified as *Acinetobacter lwoffii*. SBKVG2 showed a maximum of 99% identity towards *Prolinoborus fasciculus* strain IHBB9208. Hence the SBKVG2 was identified as *Prolinoborus fasciculus*. SBKVG3 showed maximum of 98% identity towards *Enterobacter cloacae subsp*. dissolvents strain 21FC2. Hence the SBKVG3 was identified as *Enterobacter cloacae subsp*.

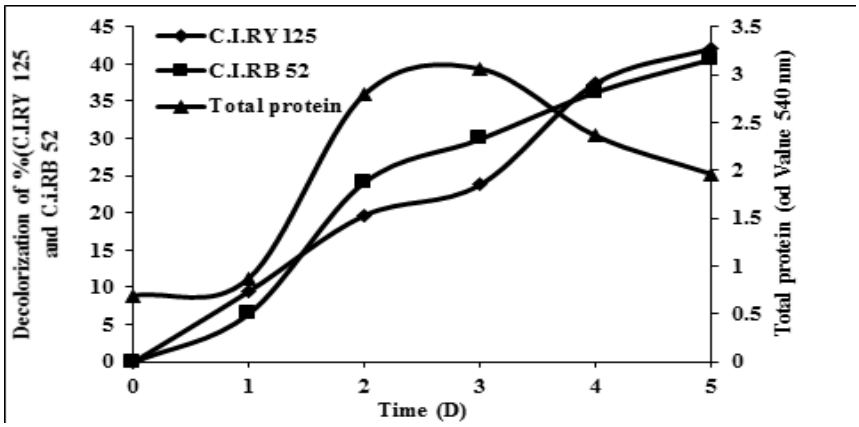


Figure 15. Decolorization of mixed reactive azo dyes (C.I.RY 125 and C.I.RB 52) in the treatment of textile effluent

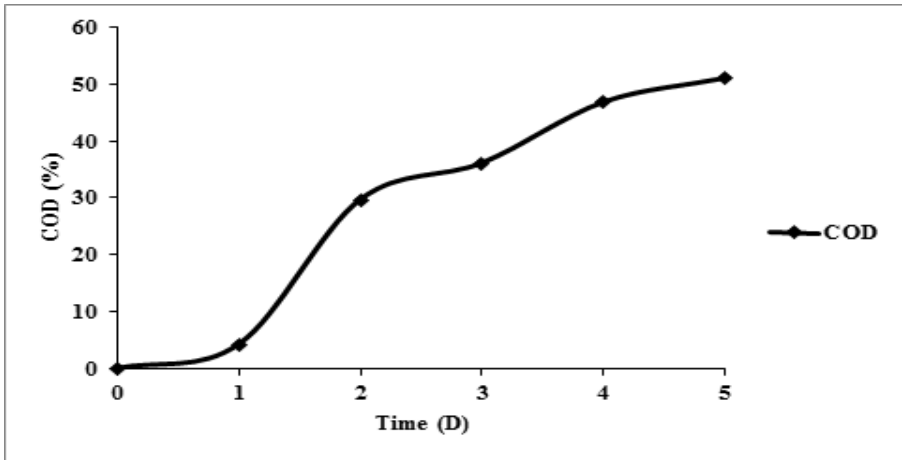


Figure 16. Removal of COD from the synthetic textile effluent wastewater

Bench-scale bioreactor studies to treat synthetic textile effluent

It was observed that the pH in textile effluent ranged from 8-9 during the process of decolorization of the reactive dyes and the values also showed that the bacterial consortium were continuously able to grow in alkaline pH which again proves the alkaline nature of the isolated bacterial consortium.

COD removal efficiencies

Percentage of maximum COD removal efficiencies were 4.25% (1st day) to 51% at the end of the 5th day respectively with an effluent. There was no significant increase in the biomass of the consortium after 5 days; this might be due to the depletion of nutrients and toxic intermediates produced during the degradation. Figure 16 shows the removal of COD from the synthetic textile effluent wastewater.

CONCLUSION

This work reports that an enriched bacterial consortium can efficiently decolorize mixed reactive azo (C.I.RY 125 and C.I.RB 52) dyes (100ppm) up to 79.6% and 77.7%, respectively in 5 days. This shows that the isolated bacterial consortium has enormous potential to degrade the textile dyes and resolve the problem of toxic dyes present in the industrial applications. Hence from the present study it could be concluded that the isolates present in the consortium contained bacterial strains like *Acinetobacter lwoffii, Prolinoborus fasciculus and Enterobacter cloacae sub sp.* which served as a best microbial source for biological decolorization of textile reactive azo dyes effluent.

C.I.R.Y 125 and C.I.R.B 52 showed a maximum decolorization at pH 8.5, which was found to be 82.9%

and 79.8% at the end of 5th day. Minimum decolorization of C.I.RY 125 and C.I.RB 52 was found to be 59.5% and 57.1% at pH 10 respectively. The optimum pH was found to be pH 8.5 for maximum removal of dye. Optimization studies on temperature indicated that only at 28˚C, enhanced growth of the bacterial consortium was seen and maximum decolorization of the mixed dyes - 81.7% (C.I.RY 125) and 82.3% (C.I.RB 52) was detected by the end of 5th day. Decolorizing activity was significantly suppressed when increasing the temperature above 37˚C until 45ºC, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 45ºC. Also, the influence of carbon sources Maltose (M), Sucrose (S), Lactose (L), Xylose (X) on the decolorization of mixed reactive azo dyes C.I.RY 125 and C.I.RB 52 at optimum concentration (100ppm) was studied. Maltose showed maximum decolorization of C.I.RY 125 (85.2%) and C.I.RB 52 dyes (79.3%) by the end of 5th day. This was followed by Sucrose, Lactose and Xylose which showed decolorization of C.I.RY 125 dye upto 79.7%, 72.9%, 70.3% and C.I.RB 52 up to 76.7%, 68.9%, 66.9% respectively by the end of the 5th day. With respect to optimization of nitrogen sources, ammonium nitrate showed the maximum decolorization of C.I.RY 125 and C.I.RB 52 dyes, which was found to be 80.8 and 81.0% by the end of 5th day respectively. This was followed by ammonium acetate, ammonium oxalate and yeast extract with C.I.RY 125 up to 74.5%, 72.0%, 65.7% and C.I.RB 52 dye up to 75.8%, 74.1%, 67.9% respectively by the end of 5th day. Maltose (M) and Ammonium Nitrate (AN) together at their optimal concentrations could further enhance the decolorization of C.I.RY 125 and C.I.RB 52 dyes, which was found to be 80.3% and 76.2% by the end of 5th day respectively.

These studies successfully demonstrate that this bacterial consortium could be employed in the enzyme study involved in the decolorisation of the reactive mixed dyes and further applied in the real time wastewater treatment.

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