Isolation, Identification and Screening of Dye Decolorizing Bacteria

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Abstract The present study deals with the isolation, identification and screening of bacterial species capable to decolorize variety of dyes. Decolorization of dyes and growth of the bacterial species are investigated. The strain ETL-1942 decolorized all the selected dyes except Remazol Brilliant Blue R, Reactive Blue H5G, Remazol Turquoise Blue G and Fast Green. In our study, we identified three groups of dyes on the basis of the pattern of dye decolorization by strain ETL-1942. The results indicate that decolorization rate is faster for the dyes of the third group than the second group. The strain can grow with all the six dyes tested, but decolorization rates are different for each dye. These results demonstrate that the strain ETL-1942 have ability to decolorize wide range of dyes and therefore further investigation for physico-chemical parameter were carried out.

Keywords: Pseudomonas, Remazol, Reactive Blue, Turquoise Blue, Fast Green

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1. Introduction

One of the major problems that humans are facing is the restoration of the contaminated environment. Textile dyes contribute as the most important environment-polluting agents. Several classes of such contaminants have been synthesized, and still new products are being synthesized now and then. The textile industry is a large water consumer and produces large volumes of contaminated water. One of such examples is the Ankleshwar Industrial Estate, Ankleshwar, Gujarat, India, which is a seriously industrialized area and produces millions of liters of improperly treated effluents that are released directly without giving proper treatment. Synthetic dyes released into the environment in the form of effluents by textile, leather, food, paper and printing industries cause severe ecological damages. Wastewater resulting from dyeing and finishing processes has an adverse impact in terms of total organic carbon, biological oxygen demand and chemical oxygen demand. Azo dyes are the main constituents of such pollution because of their wide applicability and usages, and therefore, these are present majorly in textile industrial effluents. Moreover their toxicity and resistance to degradation offer great challenge for removal technologies. In many cases the products formed after the degradation of the parent azo dye molecule are more toxic. These products are mainly in aromatic amine form. Azo dyes have been shown to be mutagenic to the human hepatoma cell line where frame shift mutation was observed [1,2]. Induction in the

micronuclei formation in human lymphocytes and in HepG2 cells after treatment with azo dye was also observed [3]. The effluent from a dye-processing plant was shown to be responsible for the mutagenic activity detected in a Brazilian river [4]. Methyl red is mutagenic in nature, and most microbial degradation studies reveal the formation of N, N-dimethyl-phenylenediamine, a toxic and mutagenic aromatic amine [5] that remains untreated in the culture [6]. Acid violet 7 has a significant ability to induce chromosome aberrations, lipid peroxidation and inhibition of acetylcholinesterase. Its toxicity increases extensively after static biodegradation with *Pseudomonas* putida, due to the corresponding azo reduction metabolites 4'-aminoacetanilide and 5-acetamido-2-amino-1-hydroxy-3,6- naphthalene disulphonic acid [7]. Therefore azo dyes are the prime attention of the researchers because of their toxicity perspectives. Several physicochemical and microbial methods have been developed for the removal and detoxification of azo dyes. However the developed physicochemical methods present several drawbacks such as high cost, high generation of sludge, high-energyrequiring irradiation methods requires a lot of dissolved O₂, etc. [8,9]. Biological methods represent more proper way of textile azo dye removal. Several microorganisms such as algae, veast, filamentous fungi and bacteria individually or in consortium are shown to degrade the azo dyes in the presence of nutrients [10-15]. However only yeast species Saccharomyces cerevisiae MTCC 463 has been shown to decolorize azo dye in plain distilled water [16]. Rapid industrialization has necessitated the manufacture and use of different chemicals in day to day life [17]. The textile industry is one of them which

extensively use synthetic chemicals as dyes. Wastewaters from textile industries pose a threat to the environment, as large amount of chemically different dyes are used. A significant proportion of these dyes enter the environment via wastewater [18]. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide [19]. The present study deals with the isolation, identification and screening of bacterial species capable to decolorize variety of dyes. Decolorization of dyes and growth of the bacterial species are investigated.

2. Materials and Methods

2.1. Chemicals

All chemicals were analytical grade and procured from Hi-media, India.

2.1.1. Dyes & Media

All the dyes (Table 1) were purchased from the market or obtained as a gift. The Glucose Peptone Yeast extract medium (pH 7) contained 1% glucose, 0.5% peptone, and 0.3% yeast extract. Sutherland (pH 7.2) medium comprised of (g/l): Succinic acid (10), Na₂HPO₄ (4), KH₂PO₄ (1.0), MgSO₄.7H₂O (0.05), CaCl₂.2H₂O (0.05), NaCl (0.2), (NH4)₂SO4 (3.0) and Yeast Extract (0.5). pHs of the media was adjusted with 1 M NaOH.

Table 1. Dyes used in the experiment

No	Name of Dyes*
1	Reactive Golden HR
2	Reactive Yellow FG
3	Remazol Brown GR
4	Remazol Orange H2R
5	Reactive Orange H2R
6	Remazol Orange 3R
7	Congo Red
8	Remazol Red H8B
9	Reactive Red 6BX
10	Remazol Magenta HB
11	Remazol Brilliant Blue R
12	Remazol Black B
13	Fast Green
14	Brilliant Green
15	Methylene Blue
16	Orange G
17	Reactive Yellow 145
18	Reactive Red HE7B
19	Reactive Red 195
20	Reactive Orange
21	Reactive Black B
22	Evan's Blue
23	Reactive Blue H5G
24	Remazol Turquoise Blue G

^{*} Stock solution of 0.1%

2.1.2. Isolation and Identification of Dye Decolorizing Bacteria

Soil samples were collected from several places of the garden. One gram of soil was suspended in 100 ml of distilled water and stirred on shaker for an hour. Soil suspension was allowed to stand for the soil particles to settle down. Supernatant was decanted and used for the

isolation and estimation of dye decolorizing bacteria. The number of dye decolorizing bacteria in garden soil of CETP (Common Effluent Treatment Plant) of Ankleshwar, Gujarat, India was estimated by three-tube most probable number (MPN) technique. The MPN technique was performed in tubes containing 10 ml GPY medium with 0.1% dye solution of Remazol Red H8B and Remazol Turquoise Blue G. 10-2 dilution of soil suspension in GPY medium was prepared and inoculated as 1, 0.1 and 0.01 ml respectively. The MPN technique was also performed with Sutherland medium as described above. All the MPN tubes were incubated at 37°C. Tubes were scored on the basis of decolorization of dyes. The number of dye-decolorizing bacteria per gram of soil was calculated following standard procedures. GPY agar plates were streaked from each positive MPN tubes and incubated at 37°C. The isolated colonies were picked up and transferred to GPY containing dye to ensure the ability of the isolate to decolorize dyes. Morphological and cultural characteristics of dye decolorizing bacteria from GPY agar plate were studied. Biochemical tests were performed to identify the dye decolorizing bacterial isolates according to Bergey's Manual of Systematic Bacteriology (Edition IV, 1984) [19].

2.2. Culture Identification by 16S RRNA Technique

2.2.1. DNA Extraction

The strain ETL-1942 (20-80 μ l) was suspended in 40 μ l MQ water. 160 μ l of 0.05 M NaOH was added and mixed well. The reaction mixture was incubated on dry bath for 45 min at 60°C and vortex intermittently. 12 μ l of 0.01 M Tris-HCl was added and the the mixture was diluted upto 100 fold and used (6 μ l) for PCR.

2.2.2. PCR and DNA Sequencing

The reaction mixture (25 µl) contained 10x reaction buffer, 25 mM MgCl2, 2 mM dNTP, 5 U/µl promega U/µl forward primer (515F Taq, GTGCCAGCAGCCGCGGTAAT-3') and 5 U/µl reverse primer (1390R 5'-AGGCCCGGAACGTATTCACC-3') and 6 µl extracted DNA. Sterile Millipore filtered deionized (6 µl) water was used as negative control. Amplification conditions were 94°C for 3 min / 94°C for 10 s, 55°C for 10 s and 72°C for 30 s x 45 cycles / 72°C for 10 min. 875 bp PCR products were electrophoresed in 1% agarose gel. PCR products were purified from agarose gel by AMPure purification method. The template is sequenced for the respective genes using the same set of primers as used for amplification of the gene. The consensus sequences of the gene so was subjected to **BLAST** (http://80www.ncbi.nlm.nih.gov.ludwig.lub.lu.se/blast/), NCBI and a phylogenetic tree was constructed using the BLAST searches.

2.3. Screening of Dye Decolorizing Bacteria

Tubes having 8 ml dye containing GPY (Table 1) was inoculated with 10% of 18 h old culture. Abiotic (uninoculated; containing dye) and biotic (inoculated; not containing dye) controls were also included. All the tubes were incubated at 37°C under static condition.

2.4. Analytical Methods

2 ml samples were withdrawn at regular time intervals and centrifuged at 9000 rpm for 20 min. Supernatant was collected and scanned (200-700 nm) using Shimadzu UV-Vis Spectrophotometer 1800. Pellets were resuspended in the same amount of distilled water and measured at A600. Residual dye was determined at γ max of each dye spectrophotometrically and used to determine the percentage of dye decolorized.

3. Results

3.1. Isolation and Identification of Dye Decolorizing Bacteria

MPN technique was used to estimate the number of dye decolorizing bacteria present in the CETP garden soil. Positive tubes exhibited decolorization of Remazol Red H8B and Remazol Turquoise Blue G as well as growth of organisms. Dye decolorizing bacteria per gram of soil using GPY medium was estimated to be 11, 000 and 430 with Remazol Red H8B and Remazol Turquoise Blue G respectively. It was estimated to be approximately 460 and 21 respectively per gram of soil using Sutherland medium. Three different types of colonies were observed on GPY agar plates streaked from positive tubes. The three bacterial strains ETL-1942, ETL-1943 and ETL-1944 were further tested individually for the decolorization of the same dyes.

Culture identification by Biochemical tests: The colonies of strain ETL-1942 were big with irregular

margin, transparent, flat and exhibited green pigmentation which turned brown upon prolonged incubation on GPY agar plate. The cells are gram negative, small rods, and motile. They are non-fermentative, nitrate, catalase and oxidase-positive. The organism was identified as *Pseudomonas spp.* on the basis of biochemical tests (Table 2) and 16S rRNA technique. Figure 1 shows phylogeny tree of the strain ETL-1942.

Table 2. Biochemical Tests of strain ETL-1942

Biochemical Tests	Results
Indole production	-
H2S production	-
Ammonia production	+
Nitrate reduction	+
Urea hydrolysis	-
Gelatin hydrolysis	-
Starch hydrolysis	-
Simmon citrate agar	+
Catalase production	+
Oxidase production	+
TSI Slant	
Butt	+
H2S production	-
Gas	-
Sugar Fermentation	
Glucose	_
Lactose	_
Sucrose	_
Arabinose	_
Sorbitol	_
Fructos	

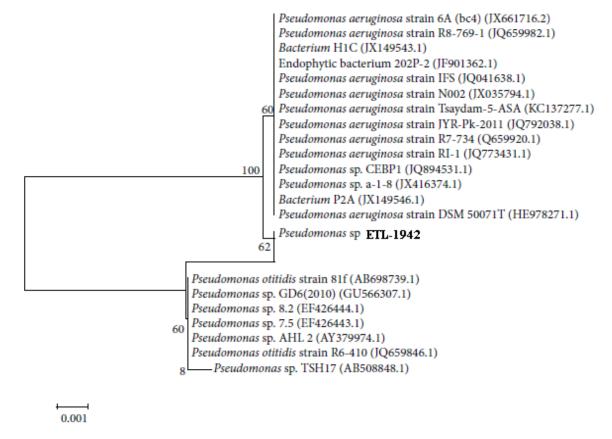


Figure 1. Phylogenetic tree of the strain ETL-1942

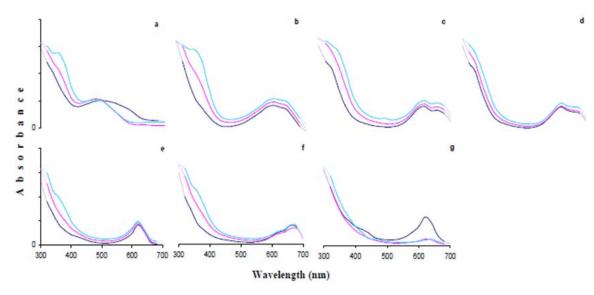


Figure 2. Changes induced in the spectra (— Control, — 24, — 48h) of first group of days, a) Congo Red, b) Remazol Brilliant Blue R, c) Reactive Blue H5G, d) Remazol Turquoise Blue G, e) Fast Green, f) Methylene Blue, g) Brilliant Green during decolorization

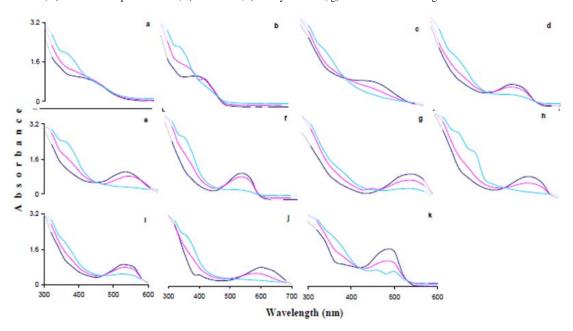


Figure 3. Changes induced in the spectra (—Control, —24, and —48h) of second group of days, a) Reactive Golden HR, b) Reactive Yellow FG, c) Remazol Brown GR, d) Reactive Orang H2R, e) Remazol Red H8R, f) Reactive Red 6BX, g) Remazol Magenta HB, h)Reactive Red HE7B,i) Reactive Red 195, j) Evan's Blue, k) Orange during decolorization

3.2. Screening of ETL-1942 for Dye Decolorizing Ability

Decolorization of twenty four dyes by bacterial strain was studied (Table 1). On the basis of dye decolorization pattern of the strain ETL-1942, three groups of dyes were observed. First group contains dyes which were not decolorized but the removal of the dye was due to adsorption on cell surface. The strain showed significant removal of Brilliant Green (88%) after 24 h incubation. The cells turned dark green in color due to adsorption of dye on the cell surface. Removal of Congo red and Methylene Blue by adsorption to cell biomass was 15 and 28% respectively but color of Methylene Blue reappeared in the medium on shaking. Negligible (8% and 5%) adsorption was observed with Remazol Turquoise Blue G and Fast Green. The strain ETL-1942 neither adsorbed nor decolorized the dyes Remazol Brilliant Blue R and

Reactive Blue H5G. In the Second group strain ETL-1942 exhibited 44-49% decolorization of Orange G, Reactive Red HE7B, Reactive Golden HR, Remazol Brown GR and Evan's Blue within 24 h. Almost 37% decolorization was observed Reactive Yellow FG, Reactive Orange H2R and Remazol Red H8B whereas 22-27% decolorization of Remazol Magenta HB, Reactive Red 6BX and Reactive Red 195 was observed after 24 h. Third group comprises of those dyes that were decolorized >75-80% within 24 h. Almost complete decolorization (95%) of Reactive Orange was exhibited by the strain Ps 33 within 24 h. The strain ETL-1942 decolorized Remazol Orange 3R, Remazol Orange H2R and Reactive Yellow 145 (83-85%) within 24 h whereas Remazol Bblack B and Reactive Black B decolorized 77%. 94% and 87% decolorization was observed after 48 h respectively. Rests of the dyes were completely decolorized after 48 h. (Figure 5). Spectral analysis (Figure 2, Figure 3 and Figure 4) indicates changes in major peak of dye for all the three groups as described above. The results indicate that decolorization rate was observed faster for the dyes of the third group than second group. On the basis of above dye decolorization pattern with the strain ETL-1942, we selected three dyes from each group for further detailed investigations. Remazol Red H8B, Reactive Red 6BX and Remazol Magenta HB were chosen from second group and studied the growth and decolorization activity of strain ETL-1942. During initial 8 h, Pseudomonas ETL-1942 decolorized Remazol Red H8B at the rate of 13 and 19 μg.h⁻¹ when the cultures were set up with 18 and 24 h old inocula respectively. Decolorization rates reached 35 and 60 µg.h⁻¹ between 8-24 h for the inocula 18 and 24 h whereas 38 and 23 µg.h⁻¹ rates were observed on further incubation of 24 h. Spectral analysis (Figure 7a & Figure 7b) indicated no decrease in the peak absorbance up to initial 8 h which decreased remarkably after 12, 16 and 20 h and the peak disappeared almost completely within 24 h; whereas it disappeared after 32 h in the cultures initiated with 24 h old inoculum. Growth of Pseudomonas ETL-1942 showed lag phase of 8 h in the cultures initiated with 24 h old inoculum but not with 18 h old inoculum. Pseudomonas ETL-1942 decolorized Reactive Red 6BX 85 and 77% in the GPY medium started with 18 and 24 h inocula. Decolorization rates after initial 8 h were 23 and 20 μg.h⁻¹ respectively for the cultures started with 18 and 24 h inocula. The rates increased to 89 and 68 μg.h⁻¹ within 8-28 h and it was 58 and 37 μg.h⁻¹ between 28-48 h. Spectral changes (Figure 7c & Figure 7d) showed that the peak absorbance decreased faster for the 18 h old inoculum in decolorization medium than the 24 h old. Growth pattern of the strain ETL-1942 was observed to be similar in the medium containing RR6BX as in the RRH8B. Decolorization reaction initiated with 18 and 24 h old inocula decolorized Remazol Magenta HB 82 and 78% respectively after 48 h. Decolorization rates were 8 and 34 µg.h⁻¹ during initial 8 h. These rates increased to 69 and 46 µg.h⁻¹ and remained steady throughout the

decolorization with 18 and 24 h old inocula. Spectral analysis showed gradual decrease in the peak absorbance. Pseudomonas ETL-1942 grew exponentially during initial 8 h in the decolorization medium inoculated with 18 h old inoculum whereas a lag phase of 12 h was observed with 24 h inoculum age (Figure 7e & Figure 7f). The three dyes Remazol Orange H2R, Remazol Orange 3R and Remazol Black B of the third group were studied in detail (Figure 2). No lag phase in the decolorization of ROH2R was observed initiated with 18 h old culture though the growth showed a lag of 4 h (Figure 7a) whereas cultures initiated with 24 h old inoculum, decolorization followed a lag of 8 h (Figure 7b). Therefore, decolorization rates for initial 8 h were 105 and 17 $\mu g.h^{-1}$ in the cultures inoculated with 18 and 24 h old inocula. During 8-20 h, the rates were 53 and 108 µg.h-1 respectively. Spectral changes showed remarkable decrease in the major peak absorbance in the GPY medium initiated with 18 h old culture than 24 h. The complete removal of the peak after 8 h in the presence of 18 h old *Pseudomonas* culture whereas it disappeared after 20 h in the presence of 24 h old culture. These cultures decolorized RBB 95 and 90% respectively. Decolorization rates during 8 h were 54 and 49 μg.h⁻¹, which increased to 96 and 122 µg.h⁻¹ between 8-20 h and 42 and 25 μg.h⁻¹ after 28 h (Figure 7c & Figure 7d). Spectral analysis indicates the complete removal of the peak after 20 h. With respect to growth of Pseudomonas ETL-1942 in the presence of RBB, a lag period was observed in both 18 and 24 h old culture. The strain ETL-1942 decolorized RO3R 28 and 45 μg.h⁻¹ during 8 h initiated with 18 and 24 h old culture respectively. During 8-20 h, it increased to 104 and 88 μg.h⁻¹ whereas 28 and 13 μg.h⁻¹ between 20-28 h. During initial 8 h, culture inoculated with 18 h old cells directly entered into log phase whereas 24 h old culture showed a lag period but decolorization pattern in both the cases was almost observed similar (Figure 7e & Figure 7f). Spectral changes indicate complete elimination of peak after 20 h.

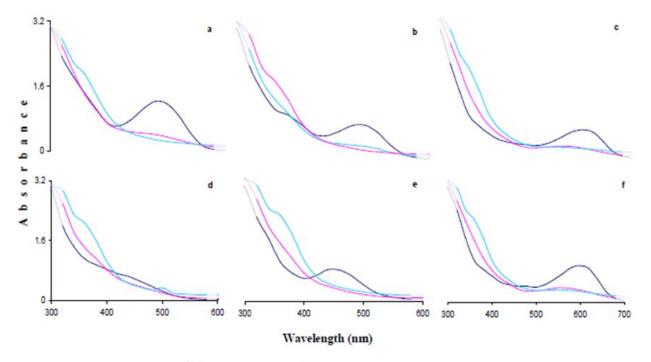


Figure 4. Changes induced in the spectra (— Control, — 24, and — 48h) of third group of days, a) Remazol Orang H2R, b) Remazol Orang 3R, c) Remazol Black B, d)Reactive Yellow 145, e) Reactive Orange and f) Reactive Black during decolorization

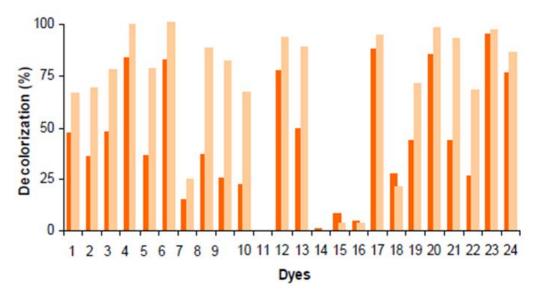


Figure 5. Decolorization of dyes after 24 and 48h by the static cultures of *Pseudomonas* **ETL-1942** at 37°C. 1) Reactive Golden HR, 2) Reactive Yellow FG, 3) Remazol Brown GR, 4) Remazol Orang H2R, 5) Reactive Orang H2R, 6) Remazol Orang 3R, 7) Congo Red, 8) Remazol Red H8B, 9) Reactive Red 6BX, 10) Remazol Magenta HB, 11) Remazol Brilliant Blue R, 12) Remazol Black B, 13) Evan's Blue, 14) Reactive Blue H5G, 15) Remazol Turquoise Blue G, 16) Fast Green, 17) Brilliant Green, 18) Methylene Blue, 19) Orange G, 20) Reactive Yellow 145, 21) Reactive Orange, 22) Reactive Red HE7B, 23) Reactive Red 195, and 24) Reactive Black 5

4. Discussion

There are number of dye-decolorizing bacteria has been reported and their characteristics reviewed [20,21,22]. Decolorization of diverse groups of dye by single bacterial species is not much studied. Pseudomonas pseudomallei 13NA and Citrobacter sp. are reported to decolorize triphenylmethane and azo dyes [23,24]. Ren et. al., [25] reported the ability of Aeromonas hydrophila to decolorize triphenylmethane, azo and anthraquinone dyes. Most of the studies show isolation of dye decolorizing bacteria from dye contaminated sites by enrichment techniques. No study observed for the enumeration of dye decolorizing bacteria present in natural environment. In the present work, we isolated a bacterial strain from noncontaminated site (garden soil) and studied its dye decolorizing ability. We also evaluated the number of dye decolorizing bacteria present in the natural sources. We therefore adopted the MPN technique to enumerate and isolate dye decolorizing bacteria present in various natural environmental samples e.g. soils and waste waters. Isolation was largely performed from the terminal positive MPN tubes. We estimated the population sizes of dye decolorizing bacteria present in the garden soil of Common Effluent Treatment Plant of Ankleshwar, Gujarat, India. The theory of the dilution culture predicts that the organisms growing in the terminal positive tubes of such dilution series were present in higher numbers in the original sample than recovered from the lower dilution. This technique allows the enumeration of only those bacteria that are able to grow on the media used. To maximize the viable count of dye decolorizing bacteria in the soil sample we used a complex (GPY) and a defined (Sutherland) medium. MPN of dye decolorizing bacteria with GPY medium was 11,000 which is higher than 430 dye decolorizing bacteria per g of soil with Sutherland medium. From the terminal positive tubes we isolated three bacterial strains ETL-1942, ETL-1943 and ETL-1944. On the basis of dye decolorizing ability, strain ETL-

1942 was selected for further study. GPY medium supported better growth and dye decolorization since it is a rich source of organic nutrients. Sutherland medium is chemically defined and contains limited amount of nutrients. On the basis of morphological, cultural, biochemical tests and 16S rRNA technique, the strain ETL-1942 was identified as Pseudomonas spp. The strain ETL-1942 decolorized all the selected dyes except Remazol Brilliant Blue R, Reactive Blue H5G, Remazol Turquoise Blue G and Fast Green. Decolorization of dyes by microorganisms takes place in two ways, either by adsorption on the microbial cells or biodegradation of dyes by the cells [21,22,23,24,25]. In our study, we identified three groups of dyes on the basis of the pattern of dye decolorization by strain ETL-1942. The first group comprises of the dyes Congo Red, Methylene Blue and Brilliant Green that are removed due to adsorption to the cells of the strain ETL-1942. These dyes deeply stained the cell surface imparting red, blue and green respectively indicating adsorption of the dyes on the cell surface. Figure 5a shows that absorbance spectra of the treated dye solution of CR, MB and BG were similar to that of the initial one. Therefore, the dye structures were not likely destroyed and no new compound was appearing during the process of dye removal. Eleven dyes comprising the second group are decolorized slowly i.e. 22-49% during 24 h and 66-94% decolorization within 48 h. The third group comprises of the dyes Remazol Orange H2R, Remazol Orange 3R, Remazol Black B, Reactive Yellow 145, Reactive Orange and Reactive Black B that are decolorized rapidly within 24 h by ETL-1942. To study the pattern of dye decolorization of the strain ETL-1942, Remazol Red H8B, Reactive Red 6BX and Remazol Magenta HB from the second group and Remazol Orange H2R, Remazol Orange 3R and Remazol Black B from the third group were selected for detailed investigation. The results indicate that decolorization rate is faster for the dyes of the third group than the second group. The strain can grow with all the six dyes tested, but decolorization rates are different for each dye. Similar observation was

also made by Paszczynski *et. al.*, [26]. Figure 7 display the UV-Vis absorbance spectra for, during different periods of decolorization. The absorbance peak at 480, 492, 596, 509, 540 and 557 nm gradually disappeared. Chen *et. al.*, [27] reported that as the dye is reduced, the broth returns to its original color. Similar results are observed with the ROH2R, RO3R, RBB and RRH8B dyes. The broth of the test and the biotic control were bluish green as the cells of the strain ETL-1942 produce pigment. Growth pattern of the strain ETL-1942 was almost similar in the presence of all the six dyes. Decolorization rate was

faster for ROH2R, RO3R and RBB than RRH8B, RR6BX and RMHB. A slower decolorization rate was attributed to higher molecular weight, structural complexity and the presence of inhibitory functional groups like –NO₂ and – SO₃Na in the dyes [28]. Several studies have recently demonstrated decolorization and degradation of structurally different dyes by bacteria [29,30,31]. These results demonstrate that the strain ETL-1942 have ability to decolorize wide range of dyes and therefore further investigation for physico-chemical parameter were carried out.

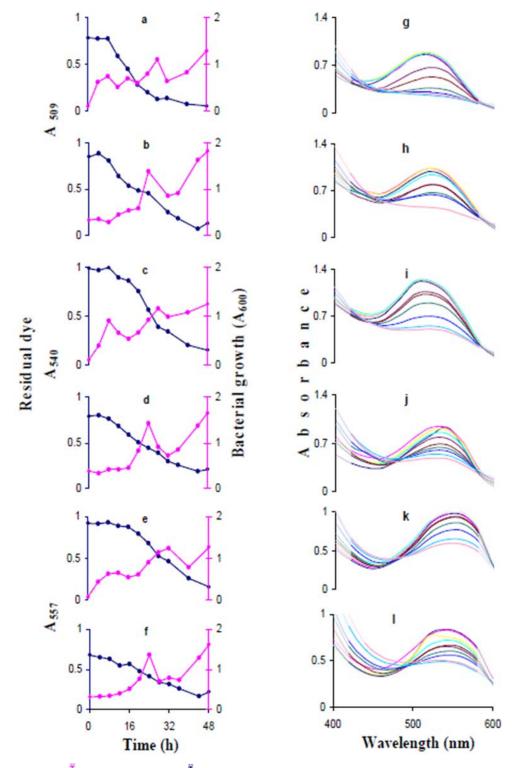


Figure 6. Change in biomass (1) and the decolorization (1) of dyes (a & b) Remazol Red H8B, (c & d) Remazol Red 6BX and (e & f) Remazol Magenta HB by the static cultures initiated with 18 and 24h old inocula and the graphs on the right depict spectral changes (— C, — 0, — 4, — 8, — 12, — 16, — 20, — 24, — 28h and — 32 h) of the same dyes associated with the decorization

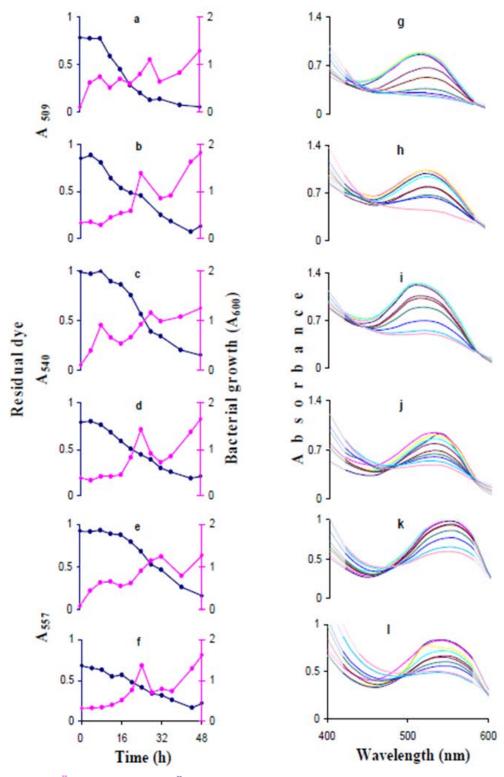


Figure 7. Change in biomass (1) and the decolorization (1) of dyes (a & b) Remazol Orange H2R, (c & d) Remazol Black 6BX and (e & f) Remazol Orange 3R by the static cultures initiated with 18 and 24h old inocula and the graphs on the right depict spectral changes (— C, — 0, — 4, — 8, — 12, — 16, — 20, — 24, and — 28h) of the same dyes associated with the decorization

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