Running title: Assessment of dye degradation by bacteria

**Biodegradation of Three Azo and One Phthalocyanine Dyes Using Bacterial Isolates Obtained from Local Textile Industry Drain**

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**Novelty Statement**

Relatively new genera in degradation studies like, Jeotagalicoccus, Comamonas and Aeromonas showed promising degradation ability against tested azo dyes. Isolated Jeotagalicocus huakuii showed the best behaviour in degradation as its degradation products had the least toxic effects among other cytotoxicity tested bacteria.

**Abstract**

Textile industry is posing serious threat to the environment as it discharges a huge number of untreated dyes. Among them azo dyes are of main concern due to their extensive use and carcinogenesis. To study the microbial degradation of four textile azo and phthalocyanin dyes: C.I reactive black 5, C.I reactive yellow 145, C.I reactive red 195 (azo dyes) and C.I reactive blue 21 (phthalocyanine dye) current study isolated and identified thirty five bacteria and one yeast from textile industry wastewater. Among them five bacteria were 16S rRNA sequenced using Macrogen facility, Korea. Isolated bacteria, belonging to *Bacillus, Comamonas, Jeotagalicoccus, Staphylococcus, Moraxella, Escherichia, Exiguobacterium, Cedecea, Kluyvera, Actinobacillus, Aeromonas, Clavibacter, Solibacillus, Paenibacillus, Micrococcus and Corynebacterium* genera, were tested to tolerate and degrade aforementioned four dyes. To study the tolerance level of bacteria against each dye, maximum 1000 ppm dye was used in the culture medium. Most of the bacteria showed maximum dye tolerance at 1000 ppm and grew well in 1000 ppm dye concentration. To study dye degradation, absorbance of dye solutions before and after bacterial treatment were recorded using UV-visible spectrophotometer and decreased dye absorbance in the visible range of light (400 nm-700 nm) was suggestive of dye degradation. On the basis of percent removal of four (black 5, blue 21, yellow 145, red 195)dyes after 5 days of incubation, *Jeotagalicoccus huakuii* (83%, 49%, 84%, 85%) *Comamonas aquatica,* (79%, 42%, 83%, 87%) *Bacillus subtilis,* (84%, 41%, 82%, 85%) *Moraxella* sp. (82%, 28%, 81%, 77%)and *Aeromonas veronii* (73%, 30%, 80%, 76%)was ranked first, second, third, fourth and fifth. Cytotoxicity of degraded products of dyes was determined and results showed the variable decrease or increase in cytotoxicity. Current study suggests that there are a number of bacteria which have the potential to degrade number of dyes and can be exploited for xenobiotics removal.

**Key words:** Microbial degradation;Wastewater; C.I reactive black 5; C.I reactive yellow 145; C.I reactive red 195; UV spectrophotometer

**Introduction**

In a country like Pakistan there is a need of an intense wastewater treatment system where wastewater pollution is becoming a big challenge day by day. According to the reports there is little to no considerable domestic and industrial wastewater treatment system throughout the country except in some cities, just up-to primary levels. Besides domestic wastewater, industrial waste water has also been discharged tremendously to environment without any proper treatment by the industry because of lack of incentives (WWF, 2007). Pakistan’s worse condition can be assessed by the facts that in Khyber Pakhtun Khawah province industrial effluents having extreme pollutants are discharged untreated in the river Kabul (SOE, 2005). Whereas in whole Sindh province out of 34 sugar industries only two have installed the wastewater treatment plants (SOE, 2005). Only fertilizer sector (UNIDO, 2000) have been reported to invest significantly for wastewater treatment plants. In Karachi there are two biggest industrial states of Pakistan (Sindh industrial and trading state and Korangi industrial and trading state) both of them do not have any kind of waste water treatment plant. Karachi contributes 70% to the Pakistan’s industry and discharges that 70% effluent directly to the Arabian Sea. In Punjab, in the Lahore city only 3/100 industries treat their hazardous chemical discharges (UNIDO, 2000).

It has been reported that about 2000 million gallons wastewater is being produced in Pakistan on daily basis and this wastewater is drained into nearby water bodies after little to no treatment (Pak-SCEA, 2006). There is no biological (secondary) treatment system in any of the cities except Karachi and Islamabad which can treat their wastewater discharge only less than 8 %. Prevailing primary treatment at low scale is extremely insufficient. In this scenario, there is a serious need to remediate the pollutants in cost effective manners by first improving primary treatment (physical removal) and then establishing secondary treatment (biological removal/ bioremediation) of contaminants which are xenobiotic in nature. Bioremediation has great potential to clean up such pollutants. Genus Zoogloea is the example of bacteria which are the members of bio-remediators help to make flocs of organic matter (settling down the pollutants) in secondary treatment system (Tortora *et al.,* 2008).

Biodegradation and bioremediation is a natural process in which microbes break, reduce and simplify the harmful waste materials and use resulting byproducts for their routine metabolic activities (White *et al.,* 2006). Beside numerous compounds dyes are among the most imperative compounds which discharge from the textile industry as waste (Phugare *et al.,* 2011). Azo dyes are being widely used in the textile sector due to their brighter colors and intense shades (Wang *et al.,* 2009) but their complex aromatic ring structures with one or more (–N=N–) bonds make them hard to degrade (Vandevivere *et al.,* 1998). Their persistence in the water bodies may lead to serious environmental and health issue. These dyes disturb the photosynthesis of aquatic plants and reduce the oxygen content of water. Serious health issues like carcinogenesis and mutagenesis, allergies and dermatitis are also caused by these recalcitrant dyes. Their by-products like aromatic amines are responsible for disturbed blood formation (Carmen and Daniela, 2012).

There are some ways to remediate the dyes from the environment like coagulation and flocculation but these processes need chemicals in bulk leaving behind a huge amount of sludge. Another way is enzymatic dye degradation which is also of limited use because of high cost, low enzymatic stability and product inhibition (Husain, 2010). Many scientists are looking forward towards novel microbes for energy efficient and cost effective bioremediation of recalcitrant dyes. Many researches have been conducted but still there is a need of searching new environment friendly microbes which are highly specialized for removal of a vast variety of dyes without being exhausted.

In current study four reactive dyes have been used for remediation study. One dye (reactive blue 21) is of phthalocyanine group and three dyes (reactive yellow 145, reactive red 195 and reactive black 5) belong to azo dye group. Among them reactive red 195 and reactive yellow 145 contains one azo bond whereas reactive black 5 contains two azo bonds (Aksakal *et al.,* 2010; Kazi *et al.,* 2016; El Bouraie *et al.,* 2016)

Textile industrial wastes samples were obtained along the main Paharang drain in Faisalabad, which receives the huge amount of textile effluent water and even domestic waste water. Samples of water and sludge were collected at 0 meter, > 10 meters and > 1000 meters away from the industrial outfall along the main drain. Only one soil sample was collected at 0 meter. All water samples were mixed to make composite sample and same was done for sludge samples. Besides sampling, temperature and pH of effluent were also measured using thermometer and digital pH meter. Samples in sterile glass flasks and beaker were transported to lab within two hours for microbiological testing. Electrical conductivity (EC) and total dissolved solids (TDS) were also measured in lab (APHA, 1998). EC values were measured using EC meter and TDS values were measured using the formula: TDS = EC × 0.6

**Materials and Methods**

**Microbiological Analysis**

**Isolation and identification:** Ten-fold dilutions were made for wastewater, sludge and soil in normal saline test tubes (0.9 % solution of NaCl). Using 8 tubes for each sample (water, sludge and soil) a total of 24 tubes were used having 9 ml normal saline in each tube. Ten-fold serial dilutions of water, sludge and soil samples were made in individual series of tubes by adding 1g of soil or 1ml of water and 1ml of sludge. Successive dilutions were made by collecting 1ml from previous tube and adding 1 ml to next tube to form 10-1 to 10-8 dilutions. Taken from test tubes 0.1 ml of diluted samples were inoculated separately on a series of respective petri plates using spread plate method. Plates were then placed in incubator for 24 hours at 37ºC for bacterial growth. After 24 hours of incubation bacterial colonies were counted as cfu/ml and later purified by streak plate method. Pure bacterial and yeast isolates were then undergone through standard microbiological testing system to get identified (Cappuccino and Sherman, 2014).

**Standard microbiological identification:** For the identification of bacteria, number of tests were performed which have been described in “Microbiology A Laboratory Manual” include: Gram’s staining, spore staining, catalase test, oxidase test, indole test, methyl red test, citrate utilization test, blood hemolysis test, motility test, starch hydrolysis test, casein hydrolysis test, mannitol salt agar test, gelatin liquefaction test, bile esculin test and nitrate reduction test (Cappuccino and Sherman, 2014).

**Molecular identification**

Among bacterial isolates obtained in present study, five isolates were further identified by 16S rRNA gene sequencing method. Macrogen commercial company, Korea has performed the sequencing using the instruments: PCR machine: DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD), PCR product purification: Multiscreen filter plate (Millipore Corp), Sequencing Kit: Big Dye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and Sequencer: ABI PRISM 3730 XL Analyzer (96 capillary type). For polymerase chain reaction, PCR forward primer 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and PCR reverse primer 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' were used whereas for sequencing, forward sequencing primer 785F 5' (GGA TTA GAT ACC CTG GTA) 3'and reverse sequencing primers 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' were used.

**Sequencing method:** The forward PCR primer and reverse primer mentioned above were used to run PCR. Using 20 ng genomic DNA as template, PCR reaction was conducted in 30 μl reaction mixture with the *EF-Taq* (Sol Gent, Korea). Following PCR conditions were observed: initial Taq polymerase activation at 95ºC for 2 minutes then 35 PCR cycles were run with denaturation at 95ºC for 1 minute, annealing at 55ºC for 1 min, and extension at 72ºC for 1 minute. At the end PCR reaction was finished with a 10-minute step at 72ºC. Purification of amplified products was performed using multiscreen filter plate (Millipore Corp., Bedford, MA, USA). The sequencing procedure was performed using a PRISM Big Dye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing extension products were added and mixed to Hi-Di formamide (Applied Bio-systems, Foster City, CA). 5 min incubation of the mixture at 95ºC was followed by 5 min on ice and then sequencing was performed by ABI Prism 3730XL DNA analyzer (Applied Bio-systems, Foster City, CA).

**Preparation of stock solutions of dyes**

For performing the dye related tests such as determination of maximum tolerance levels and bio-degradation of dyes, first of all stock solutions of dyes were prepared. Stock solution of each dye was prepared individually in deionized water after addition of 1g of dye in 100 ml of deionized water so that 10,000 ppm concentration was obtained in 100 ml (1ml contained 100 ppm as 1ppm = 1mg/L). After making the solutions of each dye syringe filter sterilization was performed for all four dyes stock solutions using filter papers of 0.22-0.45 μm pore size and then these solutions were stored in sterilized, aluminium foil covered glass flasks at room temperature.

**Assessment of bacterial tolerance against dyes**

Pure bacterial isolates were allowed to grow in variable amounts (30-1000 mg/l) of reactive dyes such as C.I. reactive blue 21, C.I. reactive red 195, C.I. reactive yellow 145 and C.I. reactive black 5 to assess dye tolerance in bacterial isolates. Nutrient agar plates added with varying concentrations (30-1000 mg/l) of these dyes were inoculated with respective bacterial isolates and incubated at 37ºC temperature for 24-48 hours. Bacteria showing different dye tolerance levels in the form of their presence or absence of growth in different dye concentrations in petri plates were recorded.

**Assessment of bacterial ability to bio-degrade/de-color the dyes**

In each test tube having 8.7 ml of autoclaved nutrient broth, 300 μl (300 ppm) of filter sterilized respective dyes from stock solution (reactive black 5, reactive red 195, reactive yellow 145 or reactive blue 21) were added individually. The test tubes then inoculated with 1 ml of individual bacterial (1-36) isolates to make the final volume of 10 ml for each test tube. Before this degradation study, bacterial inoculum was prepared by making the suspension of bacterial growth in normal saline and the turbidity of that suspension was matched with the turbidity of 0.5 McFarland’s standard solution. All test tubes were then placed in incubator at 37ºC under static conditions for 5 and 10 days. Un-inoculated tubes having dye containing nutrient broth were incubated under same conditions for assessment of abiotic de-colorization in each dye. After incubation, bacteria were separated from the culture broth by centrifugation at the rate of 3500-4000 rpm for 20 min. Measurement of de-colorization of supernatant after removal of the bacterial cell pellet was done by UV-Visible spectrophotometer (SkanIt Software RE 4.1, Thermo Fisher Scientific Oy) at λ max 423, 523, 592 and 614 nm for reactive yellow 145, reactive red 195, reactive black 5 and reactive blue 21, respectively (Khalid *et* *al.,* 2008).

**Determination of de-colorization**

De-colorization was assessed by difference in absorbance readings with or without bacterial treatment of specific dye at its respective λ max. For this, 96 wells, polystyrene, flat bottom micro-titer plates were used with the addition of 100 μl dye solution in each well. Formula used for percentage do-colorization is as follows: De-colorization %$ =\frac{At0-Atf}{At0} $× 100, 𝐴𝑡0 is initial absorbance whereas 𝐴𝑡𝑓 is final absorbance after incubation.

**Cytotoxicity assessment of dye solutions after bacterial treatment by hemolytic activity (% age)**

Percent cytotoxicity of control and bacterial treated dye solutions was determined by the method of Powell *et al*. (2000). Three ml of human blood cells were poured gently in 15 ml sterile falcon tube and washed three times with 5 ml chilled phosphate buffer saline by centrifuging the tube each time for 5 min at approximately 3000 rpm. After washing, 180 µl of RBCs suspension and 20 µl of dye solution were mixed together in a 2 ml micro-centrifuge tube. Tube again was centrifuged for 5 minutes and 100 µl of supernatant was shifted to another Eppendorf’s tube having 900 µl chilled phosphate buffered saline for dilution. As positive control 0.1% Triton X-100 was used and for negative control PBS was used. For all other solutions of dyes (treated or untreated with bacteria), this same method was followed. On an ELISA plate reader absorbance at 576 nm was recorded. Cytotoxicity was measured as % lysis of RBCs using the following formula:

$$\% Hemolysis=\frac{Absorbance of sample-Absorbance of negative control}{Absorbance of positive control}×100$$

**Results**

**Physical parameters**

During sampling, waste water temperature ranged from 28 to 38, pH readings were ranged from 7.5 to 10.5, EC readings were ranged between 4206-8745 µS/cm and TDS readings were ranged between 2523-5247 ppm. These readings were measured in water samples only.

**Microbiological analysis**

After 24 hours incubation of plates which were inoculated with diluted water, sludge and soil samples separately by spread plate method, colony forming units (CFU/mL) were measured (Table 1). For this two types of agars were used like Nutrient agar and Sabauroud Dextrose Agar.

**Microbiological Identification:** Identification of isolates was performed using standard microbiological tests as described above; identified isolates are shown in Table 2 and Table 3 with their morphological properties.

**Molecular identification of bacteria using 16S rRNA sequencing method**

Among 36 isolates, sequencing of five bacterial isolates; *Bacillus subtilis, Bacillus velezensis, Bacillus paralicheniformis, Exiguobacterium aestuarii and Aeromonas veronii,* was performed to confirm the identification of bacteria upto species level, one representative bacterial 16S rRNA gene sequencing result is presented in (Fig. 1). Accession numbers were obtained and phylogenetic trees for each of five isolates were also constructed to show the position and relativeness with other bacteria.

**Assessment of bacterial tolerance against dyes (Tolerance level determination)**

All the bacteria, which were subjected to varying concentrations of four individual dyes upto 1000 ppm in the nutrient agar plates, showed different tolerance levels. It was seen that the most of bacteria were growing at 1000 ppm concentration. Results suggest that bacteria showing growth and tolerance against dyes at 1000 ppm can tolerate dyes even in higher concentrations (Fig. 2).

**Aerobic bioremediation of dyes and heavy metals**

All the dye degradation results were obtained after aerobic incubation without agitation. Many bacteria showed very encouraging bioremediation potential, but when closely observed the test tubes having test dyes and bacteria, the bottom of the test tubes were fully de-colored whereas at the upper portion of test tube solution little dye was seen, which was suggestive of anaerobic dye degradation. The *Aeromonas veronii* showed aerobic degradation of reactive blue 21 dye as decoloration was observed at the upper portion of nutrient broth solution in test tube (Table 4).

All the isolated bacteria were able to degrade tested dyes to different extents. The most efficient dye degrading bacteria included: *Bacillus subtilis, Comamonas aquatica, Jeotagalicoccus huakuii, Bacillus paralicheniformis, Staphylococcus sciuri, Aeromonas veronii* and *Candida* species (Table 5 and Table 6). Among them, *Bacillus subtilis* and *Staphylococcus sciuri* were observed growing in acidic pH as well. Not even *Candida* specie was able to grow in nutrient broth but was also able to show dye degradation potential in nutrient broth.

Ranking of bacteria was performed on the basis of their ability to remove dyes during 5 days (Table 7) and 10 days (Table 6) of incubation. Subsequently, ranking of bacteria was also performed on the basis of their ability to remove single dye during 10 days of incubation (Table 8).

**Hemolytic assay: determination of percentage final cytotoxicity**

Hemolytic assay was performed to determine percentage of final cytotoxicity (Table 9). Dye solutions which showed prominent de-colorization visually were selected for their toxicity evaluation.

**Discussion**

It has been previously reported that azo dye degradation occurs more affectively in anaerobic conditions (Kulla, 1981), but in present study only aerobic conditions were provided for dye degradation.

All the isolated bacteria were able to degrade tested dyes to different extents. The most efficient dye degrading bacteria included: *Bacillus subtilis, Comamonas aquatica, Jeotagalicoccus huakuii, Bacillus paralicheniformis, Staphylococcus sciuri, Aeromonas veronii* and *Candida* species. Amongst them, *Bacillus subtilis* and *Staphylococcus sciuri* were observed growing in acidic pH as well. Not even *Candida* specie was able to grow in nutrient broth but was also able to show dye degradation potential in nutrient broth.

*Bacillus subtilis* (ID: BS) in present work showed the most strong capability to degrade tested dyes during incubation of 10 days as compared to other isolates (Table 4). All tested four dyes percent degradation was the highest by this bacterial sp. in 10 days. It has already been reported that *B. subtilis* is proficient to remediate 98 % of some azo dyes by its enzyme systems like laccase, azo-reductase and peroxidase just in 20 hours (Kumar *et al.,* 2015). This bacterium also has the ability to remove sulphonated azo dyes not only by simple absorbing or adsorbing to the cell wall but by proper degradation (Mabrouk and Yusef, 2008).

*Comamonas aquatica* showed good degradation of all four dyes. Especially, it degraded the reactive blue 21 dye more efficiently than most of the bacteria (Table 7) except *Clavibacter michiganensis* who showed the highest percent removal of reactive blue 21 dye (Table 8). Furthermore, it was degrading dyes little early. It has been already described that *Comamonas* spp. can degrade large poly hydroxyalkonates (PHAs) using their secretory extracellular hydrolases. These enzymes breakdown the PHAs polymers into smaller fragments that can be easily taken up by the bacteria for utilization and assimilation inside the bacterial cells (CorreÃa, 2008; Khanna and Srivastava, 2005).

*Jeotagalicoccus huakuii,* another bacterial sp. isolated during the current study, presented very good bio-degradation potential. Visually it showed complete black dye degradation from the medium in 10 days (Table 4). This organism was shown swarming in nutrient agar rich medium. This organism is [Gram-positive](https://en.wikipedia.org/wiki/Gram-positive) coccus in shape. It is moderately halophilic (salt-tolerant) able to grow in 0–23% [NaCl](https://en.wikipedia.org/wiki/Sodium_chloride) and belongs to the family [Staphylococcaceae](https://en.wikipedia.org/wiki/Staphylococcaceae) (Guo *et al.,* 2010). It was proved to be the good candidate for bio-removal of dyes.

*Aeromonas veronii* is a Gram negative rods can be isolated from fresh water, soil and clinical sources (Sinha *et al.,* 2004). This organism has some resilient properties as it shows resistance towards antibiotics like tetra-cyclins and ciprofloxacin. It may cause pathogenesis in skin, soft tissues and gastrointestinal tract of humans and fish (Skwor *et al.,* 2014). In current study this organism showed promising degradation results for all four dyes (Table 7). It was also observed growing at low temperature around 25ºC without an incubator in winter season at room temperature.

*Staphylococcus sciurii* has been obtained from textile waste water showing good bio-remediating ability as illustrated in table of percentage dye de-colorization (Table 4 and 6). This microorganism usually found in number of locales including animals, humans and environment. This bacterium signifies some special features such as presence of multiple virulence genes and resistance genes and it further acts as source of toxin and virulence genes for other Staphylococci members. Regardless of being carrier of such traits, this microorganism is considered someway harmless (Nemeghaire *et al.,* 2014). Many genes for resistance can be exploited for the bio-degradation of certain chemicals like heavy metals (Das *et al.,* 2016) and dyes.

In the current study, *Candida albicans* was seen growing at 37ºC as yeast form having oval cells and mold like form at room temperature. This yeast was growing well in nutrient broth same like bacteria and even displayed dye (aromatic complexes) removal potential (Table 4). Many type of yeasts can consume aromatic complexes as growth substrates, but as co-metabolism they use aromatic compounds more effectively (Mörtberg and Neujahr, 1985).

During current study an isolate, named *Bacillus atrophaeus* was viewed as whitish growth with large colonies on nutrient agar plates at about 37ºC, but when placed at little high temperature exhibited the darkening of colonies from off-white colony color to dark brown color which was suggestive of black pigment production by the isolate (Table 3). In literature, *Bacillus atrophaeus* is an organism phenotypically similar to *Bacillus subtilis*, but it produces a pigment in organic nitrogen containing culture medium (Nakamura, [1989](https://www.tandfonline.com/doi/full/10.3109/07388551.2014.922915)).

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Decolorization of Fast Red by Bacillus Subtilis HM

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*Micrococcus luteus,* a microorganism with lemon yellow colored, small, round, convex colony, presenting tetrad appearance under microscope, was also isolated from textile effluent water and exhibited noticeable remediating results of dyes especially black dye (Table 8). This organism has been reported to tolerate 20 % of salt content, 5 to 12 pH range and up to 45oC temperature (Salem *et al.,* 2012). So such characteristics make this bacterium resilient to perform as bio-remediator in the un-favorable wastewater environment.

Generally, all the bacterial isolates named with xS, like ES, NS etc, were proficient in growing capably on Sabouraud’s dextrose agar (Table 1). This agar is suggested to be used for fungal growth. Its pH is adjusted about 5. So organisms with xS IDs were able to grow under extensive ranges of pH as they were collected form alkaline (textile) water (pH of 9-10) and were seen growinh in acidic medium. The pH of textile wastewater is towards alkaline, containing halo-tolerant bacteria in it (Asad *et al.,* 2007). It means microorganisms isolated from such environment have more resilient properties which make them survive in such highly polluted environment.

*Bacillus, Staphylococcus, Corynebacterium, Escherichia* and some other bacteria are among the best hydrocarbon degraders (Kafilzadeh *et al.,* 2011). These already described bacteria after analyzing their potential in current study, to remove dyes showed promising results except in case of *Corynebacterium* species which showed little growth in broth media with less degradation of dyes (Table 4). *Bacillus* species are well known for the remediation of aromatic compounds (dyes) (Cybulski *et al.,* 2003) and also manifest bio-surfactant producing abilities (Abed *et al.,* 2014). Bio-surfactant production by these bacteria is helpful to decrease the surface tension of pollutant molecules at the surface of wastewater. The bio-degradation pathways for *Bacillus,* *Corynebacterium* and *Aeromonas* species have been well described(Mrozik *et al.,* 2003). Current study results were complied with previous bio-degradation knowledge with some minor differences.

Dye solutions which showed prominent de-colorization visually were selected for their toxicity evaluation. Increase in toxicity of bacterial treated solutions may have been due to the production aromatic amines which were results of azo dye breakage or may be due to extracellular toxins secreted by certain. The increase toxicity may be due to the prolonged incubation. The decrease in toxicity of bacterial treated solutions of dyes, when compared with control group, suggested bacterial treatments were good for dye bioremediation as they not only remove the dye content but also decrease the toxic effects of the resultant products of dye degradation (Table 9). Such bacteria can be used as a potential candidate for the removal of not only azo dyes but also for the removal of more toxic aromatic amines.

**Conclusion**

Textile effluent contains numerous microorganisms which are responsible for natural bioremediation of the wastewater. Bacteria and yeast isolated from industrial wastewater were capable of degrading azo and phthalocyanin dyes affectively. Double azo dye class (reactive black 5 dye) was the easiest target for bacterial degradation whereas phthalocyanin dye (reactive blue 21) was the most difficult to be degraded. Among 36 isolates *Bacillus, Jeotagalicoccus, Comamonas, Aeromonas* and *Staphylococcus* showed very promising degradation. In context of decrease toxicity of bacterial treated products of dyes, among all these five best isolates, *Comamonas* *aquatica* was ranked first with 70 % cytotoxicity decrease, *Staphylococcus sciuri* ranked second with 65 % cytotoxicity decrease and *Bacillus subtilis* ranked third with 55 % cytotoxicity decrease.

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**Author contributions**

KT performed experiments and manuscript writing, MSM planned and conceptualized the research, MA and AK interpreted the results and critical analysis

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**Fig.1:** Representative 16S rRNA gene sequenced *Bacillus subtilis* and its phylogenetic tree (Macrogen, Korea).

|  |  |
| --- | --- |
|  |  |
|  |  |

**Fig. 2:** Y axis indicates dye concentrations in ppm and bars are indicating the bacterial growth against dye concentrations used in their culture medium for determination of tolerance.

**Table 1:** Number of colonies per mL on Nutrient Agar and Sabauroud Agar

|  |  |
| --- | --- |
| On Nutrient Agar number of colonies and CFU/mL | On Sabouraud Agar number of colonies and CFU/mL |
| Soil | Sludge | Water | Soil | Sludge | Water |
| 26 bacterial colonies on 10-4 dilution plate | 68 bacterial colonies on 10-3 dilution plate | 158 bacterial colonies on 10-2 dilution plate | 84 bacterial colonies on 10-2 dilution plate | 25 bacterial colonies on 10-2 dilution plate | 17 bacterial colonies on 10-2 dilution plate |
| 2600000 CFU/g | 680000 CFU/mL | 158000 CFU/mL | 84000 CFU/g | 25000 CFU/mL | 17000 CFU/mL |
| \_\_\_\_\_ | \_\_\_\_\_ | \_\_\_\_\_ | 14 colonies on 10-4 dilution plate | 1 fungal colony on 10-4 dilution plate covering whole plate | 2 fungal colonies on 10-3 dilution plate |

**Table 2:** Standard microbiological tests performed on isolated bacteria (I)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Isolates Name and ID | Microscopic shape + Gram’s stain | Indole test | Methyl Red test | Citrate utilization test | Catalase test | Motility test | Oxidase test | Blood hemolysis test |
| *Bacillus mycoides,* A | Rods, G+ | - | - | + | + | - | - | Less hemolysis |
| *Moraxella* species, B | Rods, G- | - | - | + | + | - | + | - |
| *Actinobacillus capsulatus,* C | Coccii, G- | - | + | + | + | - | - | - |
| *Comamonas aquatica,* D | Rods, G- | - | + | + | + | + | + | - |
| *Micrococcus variance,* E | Coccii, G+ | - | + | - | + | - | + | - |
| *Corynebacterium flavescens,* G | Rods, G+  | - | + | + | + | - | - | - |
| *Corynebacterium kutscheri,* H | Rods, G+ | - | - | + | + | - | + | Beta |
| *Clavibacter michiganensis,* I | Rods, G+ | - | - | + | + | - | + | - |
| *Bacillus safensis,* K | Rods, G+ | - | - | + | + | - | + | Alpha |
| *Exiguobacterium aestuarii,* L | Rods, G+ | - | - | - | + | + | + | - |
| *Jeotagalicoccus huakuii,* M | Coccii, G+ | - | - | - | + | + | + | - |
| *Bacillus paralicheniformis,* N | Rods, G+ | - | + | + | + | + | - | Beta |
| *Aeromonas veronii,* O | Rods, G- | + | + | + | + | + | + | Alpha |
| *Solibacillus silvestris,* S | Rods+ small chains, G+ | - | - | - | + | + | - | - |
| *Bacillus mycoides,* T | Thick rods, G+ | - | - | + | + | - | + | Beta |
| *Micrococcus luteus,* V | Tetrads, G+ | - | - | - | + | - | + | - |
| *Corynebacterium bovis,* W | Small rods, G+ | - | - | - | + | - | - | - |
| *Corynebacterium pilosum,* X | Large thin rods, G+ | - | - | - | + | - | - | - |
| *Paenibacillus residui,* Z | Thin rods, G+ | - | + | - | + | + | + | Alpha |
| *Bacillus pumilus,* AA | Rods + spores, G+ | - | - | + | + | - | + | Beta |
| *Cedecea neteri,* AS | Large rods, G- | - | - | + | + | + | - | - |
| *Bacillus subtilis,* BS | Rods + spores, G+ | - | - | + | + | - | - | Beta |
| *Bacillus mycoides,* DS | Rods + spores, G+ | - | - | + | + | - | - | Alpha |
| *Candida albicans,* ES | Oval shaped, larger than bacteria | - | - | - | + | - | - | - |
| *Kluyvera intermedia,* FS | Rods, G- | - | - | + | + | + | - | - |
| *Bacillus subtilis,* GS | Thin long rods (excessive slime), G+ | - | - | + | + | - | + | Beta |
| *Bacillus atrophaeus,* HS | Rods + spore, G+ | - | - | + | + | - | - | Beta |
| *Bacillus subtilis,* JS | Oval rods, G+ | - | + | + | + | - | + | Beta |
| *Escherichia coli,* LS | Rods, G- | + | + | - | + | + | - | - |
| *Bacillus licheniformis,* NS | Rods + spore, G+ | - | - | + | + | - | + | Beta |
| *Bacillus* species, PS | Rods + spore, G+ | - | - | + | + | - | + | Alpha |
| *Bacillus megaterium,* QS | Rods + spore, G+ | - | - | + | + | - | + | Alpha+ Swarming |
| *Bacillus licheniformis,* RS | Rods + spore, G+ | - | - | + | + | - | - | - |
| *Bacillus megaterium,* SS | Rods, G+ | - | - | + | + | - | + | Little hemolysis |
| *Staphylococcus sciuri,* TS | Coccii, (diplococci), G+ | - | --- | + | + | + | + | - |
| *Bacillus velezensis,* XS | Large Rods + spore, G+ | - | - | + | + | - | + | Beta |

**Table 3: Standard microbiological tests performed on isolated bacteria (II)**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Isolate ID | Colony color | Starch hydrolysis test | Bile Esculin test | Casein hydrolysis test | Mannitol Salt Agar test | Gelatin liquefaction test | Nitrate reduction test |
| 2 d | 5 d | A+B | A+B+Zn |
| A | white | + | - | + | - | - | + | - | - |
| B | Off-white | - | + | + | - | - | + | - | - |
| C | Off-white | - | + | + | + | - | - | - | + |
| D | Orange yellow | + | - | - | - | - | - | + | --- |
| E | Light yellow | - | - | - | - | - | - | - | - |
| G | Off-white | - | - | - | + | - | - | + | --- |
| H | White | - | + | + | - | - | + | - | - |
| I | Light orange | - | + | + | + | - | - | - | - |
| K | White | - | + | + | - | - | + | - | - |
| L | Bright orange | - | - | + | + | - | - | - | - |
| M | Off-white | - | - | - | - | - | - | - | - |
| N | Light pink | + | + | + | - | + | --- | - | + |
| O | Off-white | + | - | + | - | + | --- | + | --- |
| S | Off-white | - | - | - | - | - |  | - | - |
| T | Off-white | + | + | + | - | + | --- | - | + |
| V | Lemon yellow | - | - | - | - | - | + | - | - |
| W | Transparent | - | - | - | - | - | - | - | - |
| X | Off-white | + | - | - | + | - | - | + | --- |
| Z | Off-white | - | + | - | + | - | - | - | - |
| AA | White | - | + | + | + | - | + | - | + |
| AS | Off-white | - | + | + | + | - | - | - | + |
| BS | Off-white | + | + | + | + | + | --- | + | --- |
| DS | Off-white | + | + | + | + | + | --- | + | --- |
| ES | Off-white | - | - | - | - | - | - | - | - |
| FS | Off-white | - | + | - | + | - | - | - | + |
| GS | Off-white | + | + | + | + | + | --- | + | --- |
| HS | Off-white-black | + | + | + | + | + | --- | + | --- |
| JS | Off-white | + | + | + | + | + | --- | + | --- |
| LS | Off-white | - | - | - | + | - | - | + | --- |
| NS | Light pink | + | + | + | + | + | --- | + | --- |
| PS | Off-white | + | + | + | + | + | --- | + | --- |
| QS | Off-white | + | + | + | + | - | + | - | - |
| RS | Light pink | + | + | - | + | + | --- | + | --- |
| SS | Off-white | + | + | + | + | + | --- | + | --- |
| TS | Off-white | - | + | - | + | - | - | - | + |
| XS | Off-white | + | + | + | + | + | --- | + | --- |

--- = not determined as not required

**Table 4:** Percent decrease in dye concentration after 10 days treatment with bacterial isolates (group 2)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Isolate ID | Isolate Name | Black Dye | Blue Dye | Yellow Dye | Red Dye |
| A | *Bacillus mycoides* | 86.20 % | 12.00% | 71.75% | 80.60% |
| D | *Comamonas aquatica* | 87.30% | 40.40% | 81.53% | 84.00% |
| G | *Corynebacterium flavescens* | 70.70% | 24.46% | 77.80% | 71.49% |
| H | *Corynebacterium kutscheri* | 67.0% | 15.10% | 31.40% | 34.50% |
| I | *Clavibacter michiganensis* | 80.50% | 51.89% | 68.24% | 76.00% |
| K | *Bacillus safensis* | 62.25% | 6.00% | 29.00% | 29.30% |
| L | *Exiguobacterium aestuarii* | 85.20% | 18.16% | 73.79% | 82.90% |
| M | *Jeotagalicoccus huakuii* | 88.38% | 37.00% | 82.30% | 82.30% |
| N | *Bacillus paralicheniformis* | 89.25% | 37.80% | 75.40% | 88.00% |
| O | *Aeromonas veronii* | 79.70% | 42.00% | 63.20% | 82.80% |
| S | *Solibacillus silvestris* | 71.90% | 14.73% | 75.98% | 51.75% |
| T | *Bacillus mycoides* | 87.37% | 31.70% | 71.20% | 82.80% |
| V | *Micrococcus luteus* | 91.94% | 13.60% | 47.20% | 75.80% |
| Z | *Paenibacillus residui* | 79.31% | 41.42% | 69.90% | 68.10% |
| AA | *Bacillus pumilus* | 72.40% | 15.60% | 82.70% | 81.50% |
| AS | *Cedecea neteri* | 75.20% | 26.30% | 61.89% | 67.00% |
| BS | *Bacillus subtilis* | 90.20% | 42.90% | 84.50% | 86.50% |
| ES | *Candida albicans* | 81.80% | 31.78% | 72.90% | 73.56% |
| GS | *Bacillus subtilis* | 47.95% | 4.54% | -13.00% | 13.90% |
| HS | *Bacillus atrophaeus* | 29.00% | 8.98% | 18.39% | 14.80% |
| JS | *Bacillus subtilis* | 39.69% | 20.80% | 12.00% | 40.44% |
| LS | *Escherichia coli* | 79.78% | 30.30% | 51.50% | 42.50% |
| NS | *Bacillus licheniformis* | 73.13% | 26.20% | 49.19% | 49.89% |
| PS | Bacillus species | 33.90% | 29.70% | 78.40% | 15.60% |
| QS | *Bacillus megaterium* | 69.53% | 27.15% | 58.00% | 54.45% |
| RS | *Bacillus licheniformis* | 86.43% | 39.20% | 72.00% | 81.78% |
| SS | *Bacillus megaterium* | 49.49% | 9.91% | 15.76% | 11.00% |
| TS | *Staphylococcus sciuri* | 84.75% | 49.40% | 78.60% | 86.10% |
| XS | *Bacillus velezensis* | 56.90% | 1.00% | 5.69% | 33.60% |

**Table 5:** Percent decrease in dye concentration after 5 days treatment with bacterial isolates (group 1)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Isolate ID | Isolate Name | Black Dye | Blue Dye | Yellow Dye | Red Dye |
| B | *Moraxella* species | 82.20% | 28.60% | 81.64% | 77.93% |
| C | *Actinobacillus capsulatus* | 49.87% | 24.90% | 45.47% | 32.64% |
| D | *Comamonas aquatica* | 79.47% | 42.20% | 83.10% | 87.90% |
| E | *Micrococcus variance* | 29.85% | 16.50% | 82.75% | 43.72% |
| M | *Jeotagalicoccus huakuii* | 83.37% | 49.90% | 84.70% | 85.60% |
| N | *Bacillus paralicheniformis* | 16.87% | 30.30% | 21.76% | 07.00% |
| O | *Aeromonas veronii* | 73.14% | 30.59% | 80.90% | 76.62% |
| X | *Corynebacterium pilosum* | 44.00% | 20.40% | 32.26% | 18.45% |
| BS | *Bacillus subtilis* | 84.78% | 41.0% | 82.70% | 85.70% |
| ES | *Candida albicans* | 71.86% | 25.00% | 81.50% | 61.85% |
| FS | *Kluyvera intermedia* | 66.56% | 26.00% | 51.00% | 34.86% |
| NS | *Bacillus licheniformis* | 83.37% | 26.00% | 43.39% | 27.80% |
| TS | *Staphylococcus sciuri* | 55.30% | 26.34% | 41.93% | 22.47% |

**Table 6:** Ranking of bacteria on the basis of their ability to remove dyes during 10 days of incubation

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Isolates ranking | Black dye % removal | Blue dye % removal | Yellow dye % removal | Red dye % removal |
| 1st | *Bacillus subtilis* (BS) | 90.20% | 42.90% | 84.50% | 86.50% |
| 2nd | *Staphylococcus sciuri* (TS) | 84.75% | 49.40% | 78.60% | 86.10% |
| 3rd | *Comamonas aquatica* (D) | 87.30% | 40.40% | 81.53% | 84.00% |
| 4th  | *Bacillus paralicheniformis* (N) | 89.25% | 37.80% | 75.40% | 88.00% |
| 5th  | *Jeotagalicoccus huakuii* (M) | 88.38% | 37.00% | 82.30% | 82.30% |

**Table 7:** Ranking of bacteria on the basis of their ability to remove dyes during 5 days of incubation.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Isolates ranking | Black dye % removal | Blue dye % removal | Yellow dye % removal | Red dye % removal |
| 1st | *Jeotagalicoccus huakuii* | 83.37% | 49.90% | 84.70% | 85.60% |
| 2nd | *Comamonas aquatica\** | 79.47% | 42.20% | 83.10% | 87.90% |
| 3rd | *Bacillus subtilis* | 84.78% | 41.0% | 82.70% | 85.70% |
| 4th  | *Moraxella* species | 82.20% | 28.60% | 81.64% | 77.93% |
| 5th  | *Aeromonas veronii* | 73.14% | 30.59% | 80.90% | 76.62% |

\* *Comamonas aquatica* was ranked second because of its ability to degrade blue, yellow and red dye better than *Bacillus subtilis* after 5 days of incubation.

**Table 8:** Ranking of bacteria on the basis of their ability to remove single dye during 10 days of incubation.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Isolates ranking | Black dye % removal | Blue dye % removal | Yellow dye % removal | Red dye % removal |
| 1st | *Micrococcus luteus (91.94%)* | *Clavibacter michiganensis (51.89%)* | *Bacillus subtilis**(84.0%)* | *Bacillus paralicheniformis**(88.0%)* |
| 2nd | *Bacillus subtilis (90.20%)* | *Staphylococcus sciuri (49.4%)* | *Bacillus pumilus**(82.7%)* | *Bacillus subtilis**(86.5%)* |
| 3rd | *Bacillus paralicheniformis (89.25%)* | *Bacillus subtilis**(42.9%)* | *Jeotagalicoccus huakui (82.3%)* | *Staphylococcus sciuri (86.1%)* |
| 4th  | *Jeotagalicoccus huakui (88.38%)* | *Aeromonas veronii (42.0%)* | *Comamonas aquatica (81.53%)* | *Comamonas aquatica (84.0%)* |
| 5th  | *Comamonas aquatica (87.30%)* | *Paenibacillus residui (41.42%)* | *Staphylococcus sciuri (78.0%)* | *Aeromonas veronii (82.0%)* |

**Table 9:** Hemolytic assay showing % final cytotoxicity

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Isolates Names | Black  | Blue | Yellow | Red |
| Control | 10.289 | 10.434 | 16.014 | 8.768 |
| *Bacillus subtilis* | --- | --- | 7.174 | 10.652 |
| *Jeotagalicoccus huakuii* | 6.594 | --- | 10.362 | 7.318 |
| *Comamonas aquatic* | --- | --- | 4.710 | 9.492 |
| *Staphylococcus sciuri* | 13.478 | --- | 5.507 | --- |
| *Aeromonas veronii* | 12.753 | 10.217 | 12.101 | 7.826 |
| *Bacillus paralicheniformis* | 15.579 | 14.927 | 16.376 | 11.956 |
| *Bacillus pumilus* | --- | --- | 3.188 | 17.681 |
| *Bacillus sp.* | --- | --- | 7.898 | --- |
| *Candida albicans* | 3.840 | --- | 14.710 | --- |

--- = not determined