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*Research Paper*

# Development and Application of an Alkaliphilic, Halotolerant Bacterial Consortium for the Treatment of Azo Dyes

**Noshaba Hassan Malik[1,2*], Misbah Khan[1], Ammar Ahmed[1], Nicolette A. Zhou[3], Maliha Ahmed[2] and Naeem Ali[2]**

[1] Department of Medical Lab Technology, Ibadat International University Islamabad
[2] Department of Microbiology, Quaid-i-Azam University Islamabad 45320, Pakistan. noshaba.h.malik@gmail.com
[3] Department of Environmental and Occupational Health Sciences, University of Washington, 4225 Roosevelt Way NE Suite 100, Seattle, WA 98195-2700, USA
**Correspondence:** noshaba.h.malik@gmail.com

## Abstract:

This study presents a novel application of an acclimated alkaliphilic, halotolerant bacterial consortium for treatment of a reactive azo dye mixture. Alkaliphilic, halotolerant bacteria were isolated from pretreated garden soil amended with azo dye mixture, NaCl, and NaOH. Twenty-nine bacteria were isolated, of which three demonstrated 81-85% decolorization of 50 ppm dye mixture within 24 hours. When the three isolates were combined, the consortium demonstrated 95% decolorization. Through phenotypic and 16S rRNA analysis, the isolates were identified as Oceanobacillus oncorhynchi NHHA1, Halomonas sp. H1(2016) NHHA2 and Halomonas kenyensis NHHA3. The alkaliphilic, halotolerant consortium, demonstrated 98% decolorization of the reactive azo dye mixture (50 ppm) within 24 hours at 35°C, in mineral salt medium (pH 10, 10% [w/v] NaCl) supplemented with glucose and yeast extract (0.1% [w/v] each). The consortium showed 80-90% decolorization activity, over a wide range of pH (8-13) and NaCl concentrations (0-12% [w/v]). The consortium also exhibited an appreciable tolerance to the dye mixture (50-300 ppm). Biodegradation of the dye mixture was confirmed by characteristic band changes in the FTIR spectrum of the decolorized dye mixture. Furthermore, phytotoxicity and brine shrimp lethality assays indicated a reduction in the toxicity of the dye mixture following bacterial treatment.

**Keywords:** *Alkaliphilic, Halotolerant, Consortium, Azo dyes, Treatment*

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**Introduction:**

Reactive azo dyes represent one of the most extensively used coloring agents in the textile industry. This group of textile dyes contains a large assortment of bright colors that have remarkable fastness profiles and are relatively easy to apply. Approximately 50% of applied reactive dyes are lost to the waste stream due to their high-water solubility. Release of copious quantities of untreated and/or partially treated dye waste in receiving water bodies poses a serious threat to the environment as many textile dyes and their metabolites are toxic in nature. Dye waste adds a chemical load to the receiving waters and a majority of the dyes have been documented to have mutagenic properties, making them potentially carcinogenic). Moreover, the presence of color in receiving surface waters reduces sunlight penetration, which ultimately leads to disturbances in the aquatic communities as dissolved oxygen levels decrease due to reduced photosynthetic activity. Therefore, treatment of textile dyes is crucial to alleviate textile waste related environmental issues.

Most of the reactive azo dyes contain complex structures making them recalcitrant in nature. A variety of physical and chemical methods are used for treating dye waste, including adsorption, filtration, precipitation, coagulation, ozonation, electrochemical oxidation, and photooxidation. However, there are many disadvantages to these methods, such as inflated cost, low efficacy, and generation of secondary sludge that requires additional treatment. Biological treatment presents a more efficient and environmentally friendly approach. Bacteria are considered the best microbial option for dye waste treatment, due to their easy and rapid growth, varied oxygen requirements, and adaptive nature to harsh environmental conditions

A considerable number of investigations have been done in the field of bacterial dye waste treatment, both with pure cultures and mixed communities Despite the promising performance of individual bacterial isolates in dye decolorization, the isolates may not be applicable for treatment of actual textile wastewaters due to the complex composition of these wastewaters and the specificity of the bacterial isolates for individual dyes. Therefore, for effective and complete treatment of textile wastewaters with multiple dyes present, mixed microbial communities showing synergistic activities presents a better alternativ. Moreover, there are various reports of dye decolorizing bacteria that operate under anaerobic, aerobic, and facultative condition. However, most of the reported bacteria carry out decolorization under neutral pH and salinity free conditions. This can limit their application when used for treatment of alkaline and high salt containing textile effluents. Under these conditions, normal bacterial cells fail to perform due to impaired metabolic activities and plasmolysis. This can be overcome by the application of extremophilic (alkaliphilic and halotolerant) bacterial candidates that possess a more adaptive physiological profile.

Most dye decolorizing bacteria are isolated from dye contaminated niches, including soil, wastewaters, and sludge from industrial sites and effluent treatment plant. Adaptation to harsh environments is a promising ability for the isolation of bacteria capable of decolorizing textile dyes in alkaline and high salinity effluents. Therefore, the objective of this study was to isolate

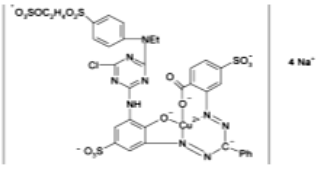
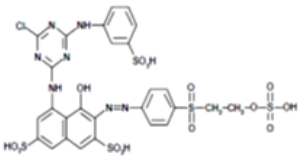
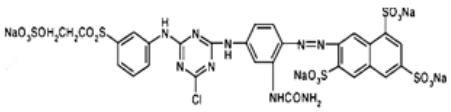
extremophilic dye decolorizing bacteria from artificially pretreated garden soil. An alkaliphilic and halotolerant consortium was developed and consisted of the isolated organisms, Oceanobacillus oncorhynchi NHHA1, Halomonas sp. H1(2016) NHHA2, and Halomonas kenyensis NHHA3. This consortium was optimized for the treatment of a reactive azo dye mixture consisting of: Reactive Blue 221 (RB 221), Reactive Red 195 (RR195) and Reactive Yellow 145 (RY 145). There are multiple reports on the application of halotolerant and/or halophilic and alkaliphilic bacteria for the treatment of different types of dyes, though few researchers have studied dye degradation under combined high pH and saline conditions. Additionally, there are only two studies where bacterial pure cultures were used to treat individual dyes under high pH and salinity conditions (. Based on the author's knowledge, this is the first report on the application of an acclimatized alkaliphilic and halotolerant bacterial consortium for the treatment and detoxification of a reactive azo dye mixture under alkaline pH and high salinity conditions.

## Material and Methods

### *Dyes, chemicals and microbiological media*

Reactive azo dyes used in this study (RB 221: $\lambda_{max}$ 615 nm, RR 195: $\lambda_{max}$ 545 nm, and RY 145: $\lambda_{max}$ 422 nm) (Table 1) were obtained from a textile unit in Lahore, Pakistan and utilized without further purification. The stock dye solution (1000 ppm; 333.33 ppm of each azo dye) was prepared by adding the individual dyes in equal proportions to distilled water, and filter sterilizing the solution (0.2 µm nylon membrane; Corning Inc., Corning, NY, USA). The stock dye solution was stored at room temperature. Microbiological media and chemicals of analytical grade were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and Oxoid (Oxoid Limited, Hampshire, United Kingdom).

**Table 1.** Chemical structures of reactive azo dyes

| Dyes | Structures | $\lambda_{max}$ (nm) | Reference |
|---|---|---|---|
| Reactive Blue 221 |  | 615 | Pajot et al. 2007 |
| Reactive Red 195 |  | 545 | Aguedach et al. 2005 |
| Reactive Yellow 145 |  | 422 | Nawahwi 2013 |

### *Medium for growth and degradation studies*

Nutrient broth and agar were used for the inoculum preparation and pure culture storage (4℃), respectively (Lapage S.P 1970). For degradation studies, mineral salt medium was prepared as previously described (Khehra et al. 2005). This medium consisted of a macronutrient and micronutrient solution. The macronutrient solution was prepared with the following composition (g/L): 3.6 $Na_2HPO_4 \cdot 7H_2O$; 1.0 $(NH_4)_2SO_4$; 1.0 $KH_2PO_4$; 1.0 $MgSO_4$; 0.01 $FeSO_4$; and 0.1 $CaCl_2 \cdot 2H_2O$. To this solution, 10 mL/L of the micronutrient solution of the following composition was added (mg/L): 10 $ZnSO_4 \cdot 7H_2O$; 3.0 $MnCl_2 \cdot 4H_2O$; 1.0 $CoCl_2 \cdot 6H_2O$; 2.0 $NiCl_2 \cdot 6H_2O$; 3.0 $Na_2MoO_4 \cdot 2H_2O$; 30.0 $H_3BO_3$; and 1.0 $CuCl_2 \cdot 2H_2O$. This mineral salt medium was supplemented with additional carbon and nitrogen sources *i.e.*, glucose and yeast extract respectively (0.1% [w/v] each). Filter sterilized stock dye solution was then added to this medium according to the required final concentration. Moreover, the medium was amended with 10% (w/v) NaCl and the final pH adjusted to 10.0 ±0.2.

### *Sampling and enrichment*

A humus rich soil sample was collected from the botanical garden at Quaid-i-Azam University in Islamabad, Pakistan. In plastic pots, 25 g of the soil sample was pretreated with 20 mL each of 10% (w/v) NaCl, NaOH at pH 10, and 1000 ppm azo dye mixture solution for the enrichment of alkaliphilic and halotolerant dye decolorizing bacteria in the soil media. With the addition of 20 mL each of the three solutions, the soil was completely flooded with water. For three months, the pots were stored in sunlight and regularly moistened with the three solutions every two days with approximately 5-10 mL of each solution added depending on the condition of the soil. The pretreated soil sample was then used as the inoculum for further enrichment of alkaliphilic and halotolerant dye decolorizing bacteria in liquid medium.

In 250 mL conical flasks, 1 g of the pretreated soil was added to 100 mL of nutrient broth medium containing 25 ppm azo dye mixture and amended with 10% (w/v) NaCl and NaOH at pH 10. The flasks were then incubated (35$^o$C) under shaking (135 rpm) and static conditions until disappearance of color. Ten (10) mL of each of the decolorized samples were successively transferred to fresh nutrient broth medium containing dye (25 and then 50 ppm) and incubated as completed previously until complete decolorization occurred. Following that, the samples were transferred to mineral salt medium (pH 10, 10% NaCl) containing 50 ppm azo dye mixture and additional carbon and nitrogen sources. The samples were incubated (35$^o$C) until complete color removal occurred.

### *Isolation and screening*

The decolorized samples (1 mL of each) were serially diluted, and 50 µL each of the diluted samples were plated on mineral salt medium agar amended with 50 ppm azo dye mixture (pH 10, 10% NaCl). The plates were incubated (35℃, 24 hours), then morphologically distinct bacterial colonies were picked and purified by the streak plate method. Pure cultures were then screened for their ability to efficiently decolorize azo dyes. Inocula were prepared by transferring a single colony to nutrient broth medium using a sterile inoculation loop followed by incubation (35℃) until mid-log phase (optical density at 600 nm [$OD_{600}$] was 0.6). Ten (10) mL of the prepared inocula were added to salt medium (90 mL) containing 50 ppm azo dye mixture (pH 10, 10% NaCl) and incubated (35$^o$ C, 24 hours) with and without agitation. The initial absorbance of each sample was recorded at 543 nm ($\lambda_{max}$ of the dye mixture) using a UV-Vis Spectrophotometer (Agilent 8453; Agilent Technologies, Santa Clara, CA, USA). After 24 hours, 1.5 mL of each treated sample was aseptically collected, centrifuged (12,000 rpm, 10 minutes, room temperature), and

the absorbance was measured. The decolorization percentage was calculated using the following formula (Sani and Banerjee 1999):

$$Decolourization\ (\%) = \frac{(I - F)}{I} \times 100 \qquad (Eq\ 1)$$

where I is the initial absorbance of the colored sample and F is the final absorbance of the decolorized sample. Uninoculated medium containing the dye was used as the abiotic control, and dye-free medium was used as the blank.

### *Microbial identification*

The cultural and biochemical properties of the selected isolates were studied. Isolates were characterized by 16S rRNA gene sequencing. Genomic DNA of the isolates was extracted by CTAB method (Andreou 2013). For 16S rRNA sequencing, samples were sent to Macrogen Inc. (Seoul, Korea), and sequenced using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′) bacterial primers. Sequences were edited using BioEdit 7.2.6 and analysed for similarity by submission to the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned and phylogenetic analysis was carried out using the neighbour joining method in MEGA6 software.

### *Consortia development and screening*

To ascertain the effect of the mixed culture on the decolorization efficiency (50 ppm dye mixture, pH 10, 10% NaCl), equal volumetric proportions of selected alkaliphilic and halotolerant isolates in the mid-log growth phase were mixed in different combinations and incubated under static condition (35℃, 24 hours). Four different combinations were investigated: NHHA1/NHHA2, NHHA1/NHHA3, NHHA2/NHHA3, and NHHA1/NHHA2/NHHA3. The decolorization percentage was computed using Equation 1. The most efficient combination was then subjected to further investigation.

### *Effect of culture conditions on dye mixture decolorization*

Decolorization activity of the selected consortium was monitored under different culture conditions by modifying the carbon source (lactose, sucrose, sorbitol, glucose, starch at 0.1% [(w/v]), nitrogen source (peptone, yeast extract, ammonium sulfate, sodium nitrite, potassium nitrate at 0.1% [(w/v]), temperature (25, 30, 35, 40, 45℃), dye concentration (50, 100, 200, 300, 400, 500 ppm), pH (8-13), and salt tolerance (0, 2, 4, 7, 9,11, 12% [w/v] NaCl). All experiments were conducted without agitation for 120 hours at 35°C.

### *Fourier transform Infrared (FTIR) spectral analysis*

Samples taken before and after treatment of the 50-ppm azo dye mixture with the bacterial consortium were subjected to FTIR analysis (spectrum 65 FTIR spectrometer equipped with ATR; Perkin Elmer, Waltham, MA, USA) and the transmission spectra were studied. Dye metabolites were extracted using ethyl acetate followed by drying over anhydrous $Na_2SO_4$ and evaporation in a rotary evaporator. The analysis was carried out in the mid IR region of 650-4000 $cm^{-1}$ with 16 scan speed.

### *Toxicity analysis of treated dye samples*

The toxicity of samples taken before and after treatment of the 50 ppm azo dye mixture with the bacterial consortium, were compared through the phytotoxicity assay using *Raphanus sativus*

seeds (Turker and Camper 2002). Moreover, cytotoxicity of the samples was tested using *Artemia salina* larvae according to Meyer et al. (1982).

### *Statistical analysis*

All experiments were carried out in duplicate. Mean, standard error, and Student's t-test (with a significance threshold of 0.05) were calculated using MS-Excel 2016.

## Results and Discussion

### *Isolation and screening of alkaliphilic and halotolerant, reactive azo dye mixture decolorizing bacteria*

A total of 29 bacteria were isolated from the pretreated garden soil through enrichment in broth cultures. To select the efficient decolorizers, isolates were screened by incubating for 24 hours under static and shaking conditions, and isolates showed more efficient color reduction without agitation (Fig. 1) ($p$<0.00001, one-tailed Student's t-test). Of the 29 isolates, 13 decolorized 70-79% under static conditions and up to 50% under shaking conditions; 7 decolorized 60-69% under static conditions and less than 50% with shaking; and 6 isolates decolorized 50-59% under static conditions and less than 50% with shaking. The remaining 3 isolates (NHHA1, NHHA2, and NHHA3) showed an 81-85% color reduction under static conditions and a 55-66% color removal with shaking. As efficient color removal was observed under both tested conditions, these three isolates were used for further experimentation, and these experiments were conducted under static conditions. The ability of the selected isolates to grow and decolorize under both aerobic/shaking and anoxic/static conditions, indicates the facultative nature of the bacteria.

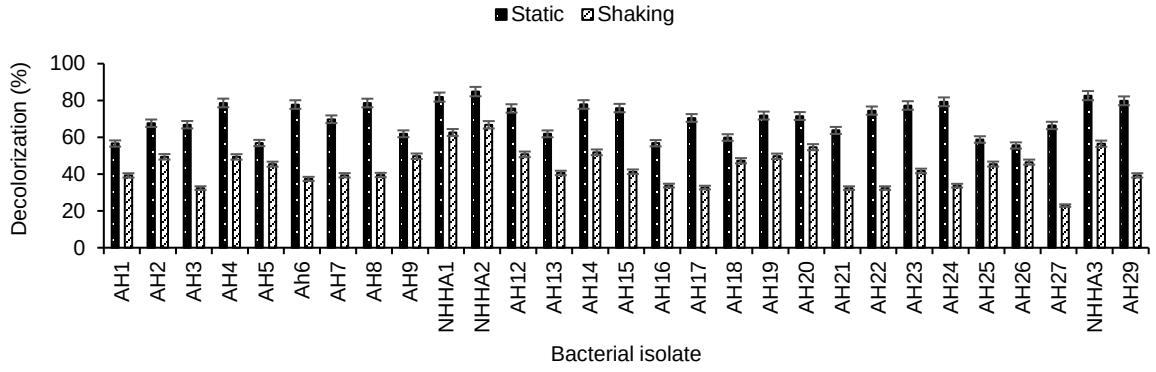



**Fig. 1** Comparison of alkaliphilic, halotolerant bacterial isolates for the decolorization of a 50-ppm reactive azo dye mixture (10% [w/v] NaCl, pH 10, 35℃) under static and shaking conditions (24 hours)

The more efficient color reduction seen under static conditions is in accordance with available literature. For example, Prasad and Rao (2013) reported 95% decolorization of 5000 ppm Direct Red 22 in 4 hours by *Bacillus cohnii* (alkaliphilic bacterium) under static conditions, but only 7% with shaking. Additionally, Kumar Garg et al. (2012) observed 69% decolorization of 100 ppm Orange II (100 ppm) in 96 hours by *Pseudomonas putida* SKG-1 under static conditions, and 36.5% decolorization with shaking. Similar findings were reported by Wang et al. (2009) with 96% and 13% color reduction of 200 ppm Reactive Red 180 in 36 hours by *Citrobacter* sp. CK3 under static

and shaking conditions, respectively. The low color removal efficiency under aerobic conditions could be attributed to the competition between oxygen (a strong terminal electron acceptor) and the azo bond for the oxidation of NADH (Chang et al. 2001).

***Microbial identification***

Morphological and biochemical properties of NHHA1, NHHA2, and NHHA3 are summarized in (Table 2). NHHA1 was a Gram-positive rod-shaped bacterium, and NHHA2 and NHHA3 were Gram negative rod shaped bacteria. Partial 16S rRNA gene sequence analysis of NHHA1, NHHA2, and NHHA3 showed 99% homology to *Oceanobacillus oncorhynchi, Halomonas* sp. H1(2012), and *Halomonas kenyensis,* respectively. MEGA6 was used for the phylogenetic analysis of isolates (Fig. 2), and the sequences were submitted to GenBank under the following accession IDs: KX355482, KX355483, and KX355484 for NHHA1, NHHA2, and NHHA3, respectively.

**Table 2.** Morphological and biochemical properties of alkaliphilic, halotolerant dye decolorizing isolates

| Properties | Bacterial isolates | | |
|---|---|---|---|
| | **NHHA1** | **NHHA2** | **NHHA3** |
| Colony morphology (on nutrient agar) | Small, round whitish, raised, shiny colonies | Small, round opaque colonies | Whitish, smooth colonies with entire margins |
| Gram's reaction | + | − | − |
| Morphology | Rods | Rods | Rods |
| Glucose | + | + | + |
| Sucrose | + | + | + |
| Lactose | + | + | + |
| Citrate | + | ± | − |
| $H_2S$ production | − | − | − |
| Indole production | + | + | − |
| Methyl red | − | − | − |
| Voges Proskauer | − | − | − |
| Motility | + | + | + |
| Catalase | + | + | + |
| Oxidase | + | − | − |
| Endospore formation | + | − | − |

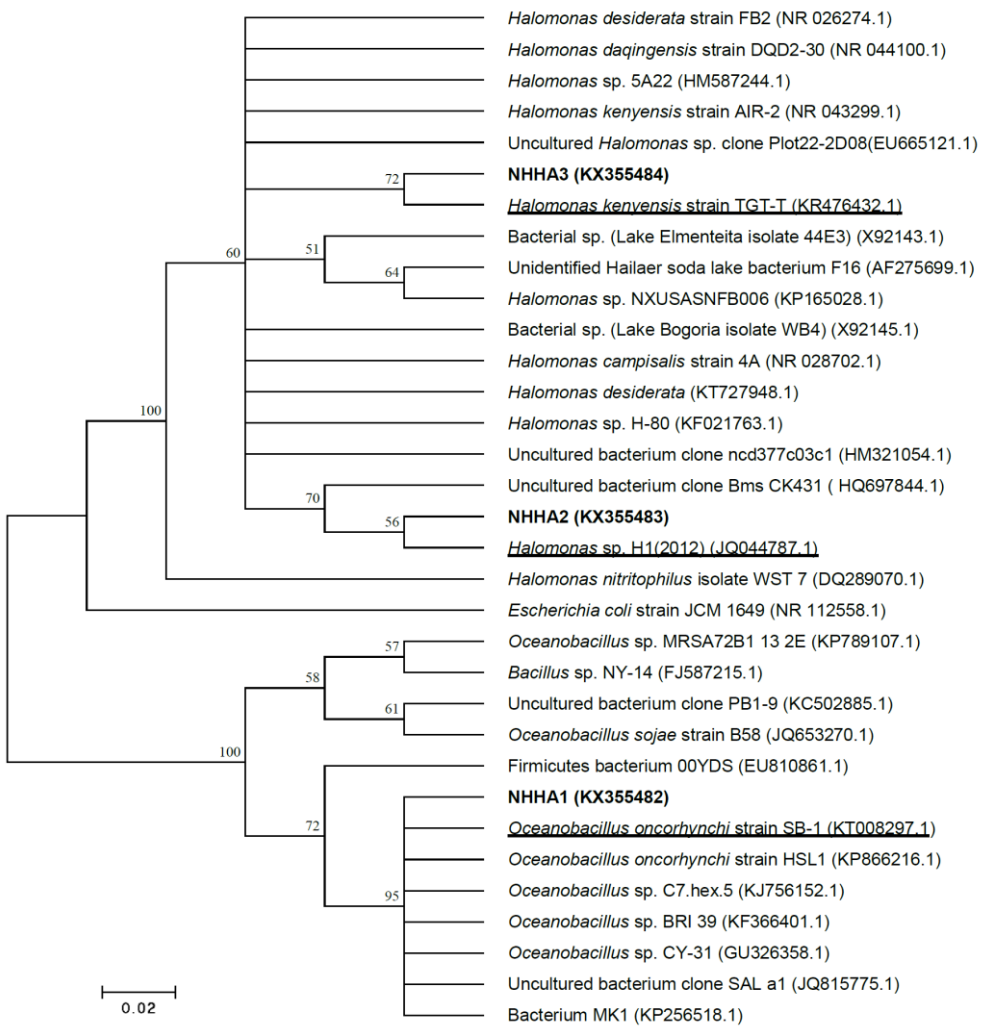



**Fig. 2** Phylogenetic analysis of selected alkaliphilic, halotolerant reactive azo dye mixture decolorizing bacterial isolates. Evolutionary analysis was completed using the Neighbor-joining method in MEGA6. The bootstrap tree consensus tree was inferred from 1000 replicates. Branches corresponding to less than 50% bootstrap replicates were collapsed. All positions containing gaps and missing data were eliminated

### *Consortium development and screening*

Selected isolates were mixed in different combinations to study the effect of mixed cultures on the color removal performance (50 ppm dye mixture, 10% [w/v] NaCl, pH 10). The decolorization ability of two of the four combinations (NHHA1/NHHA2 and NHHA1/NHHA3) was not statistically different to that of the pure cultures ($p$=0.10 and 0.20, respectively). The third combination (NHHA2/NHHA3) showed a ~4.0% increase in the decolorization capability when compared to the decolorization capability of NHHA2 and NHHA3 alone ($p$=0.001). When the 3 isolates were combined (NHAA1/NHAA2/NHAA3), there was an ~11% rise in the decolorization when compared to the decolorization of each isolate alone ($p$=0.00006). With the combination of NHAA1, NHAA2, and NHAA3, 95% color removal was observed in 24 hours (Fig. 3). As this consortium was found to be more efficient for color removal, it was used for

further experiments. Previous studies have also reported enhancement of color removal capability when using mixed cultures. Khehra et al. (2005) observed faster decolorization of 20 ppm Acid Red 88 by a consortium of four bacteria (*Bacillus cereus*, *Pseudomonas putida*, *Pseudomonas fluorescence* (BN-5) and *Stenotrophomonas acidaminiphila*) when compared to the individual isolates. To achieve the same level of decolorization that the consortium achieved in 24 hours, individual isolates required 60 hours. Saratale et al. (2009) also reported similar findings, with the decolorization of 50 ppm Scarlet R at a faster rate when using consortium-GR than when using the pure cultures *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus*. The improvement in the color removal percentage could be due to synergistic activities of the bacteria in mixed communities where different enzymes are available to carry out a given function in the degradation pathway (Ali 2010).

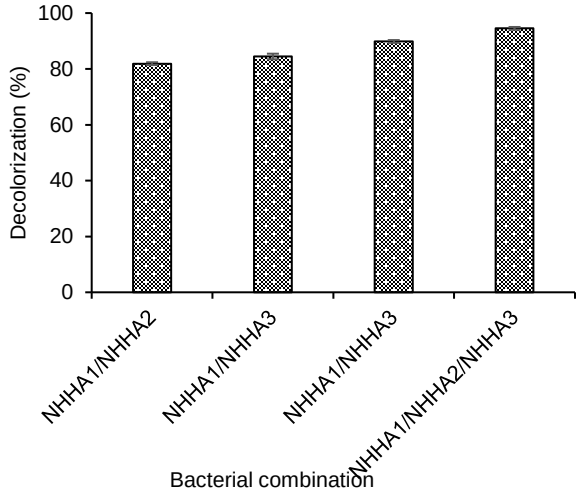



**Fig. 3** Effect of different combinations of selected alkaliphilic, halotolerant bacterial isolates on the decolorization of 50 ppm reactive azo dye mixture (10% [w/v] NaCl, pH 10, 35℃) under static condition (24 hours)

**Effect of culture conditions on dye mixture decolorization**

***Additional carbon sources***

During dye decolorization, the additional carbon source provides bacteria with required energy. Moreover, it enhances color removal by facilitating azo bond cleavage by acting as an electron donor (Moosvi et al. 2007). To test this hypothesis, the decolorization efficiency of the developed alkaliphilic and halotolerant consortium for the reactive azo dye mixture (50 ppm dye mixture, 10% [w/v] NaCl, pH 10) was tested without the addition of another carbon source. As expected, a low color removal (24.67% after 120 hours) was observed in the absence of an additional carbon source. Next, color removal performance was monitored in the presence of different additional carbon sources *i.e.* lactose, sucrose, sorbitol, glucose and starch added at 0.1% (w/v), and the presence of the any additional carbon source resulted in improved decolorization ($p$<0.00001) (Fig. 4a). Compared to the other tested carbon sources, glucose was found to be the most effective ($p$<0.00001), with a 95.43% color removal recorded after 72 hours. This was followed by sucrose, sorbitol, and lactose with a decolorization after 120 hours of 82.24%, 76.99%, and 68.21%,

respectively. Starch was found to be the least effective of all carbon sources as only 37.45% color removal was observed after 120 hours ($p$<0.00001). Similar results were reported by Jain et al. (2012), where glucose (0.1% w/v) was found to be most effective carbon source for decolorization of 200 ppm Reactive Violet 5 by an indigenous mixed culture. Glucose is one of simplest and easily utilizable carbon and energy sources for bacteria and is therefore, usually the most preferable additional carbon source for azo dye decolorization (Solís et al. 2012).

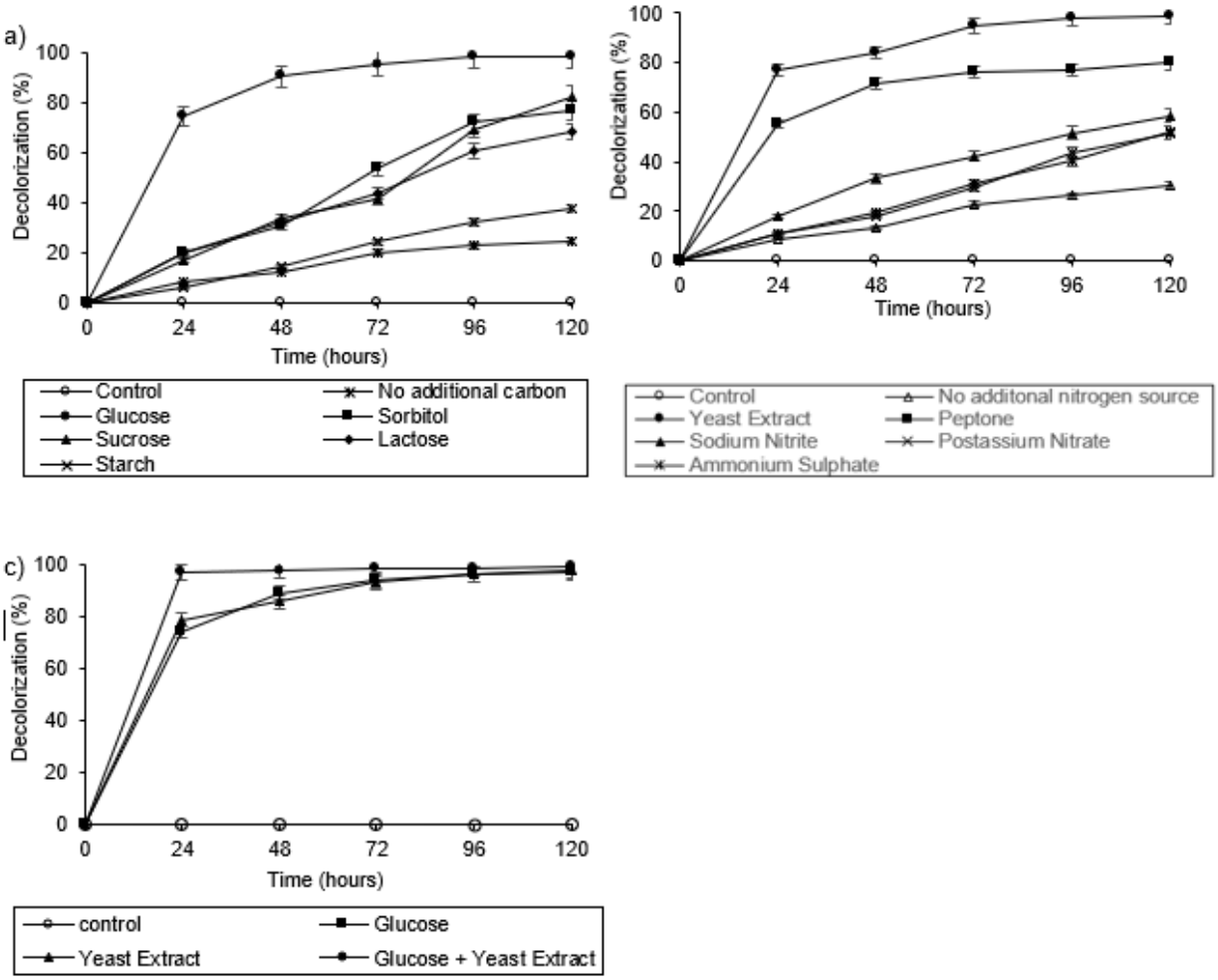



**Fig. 4** Effect of supplemental agents on decolorization of 50 ppm reactive azo dye mixture (10% [w/v] NaCl, pH 10, 35℃) by alkaliphilic, halotolerant bacterial consortium; **(a)** Effect of additional carbon sources (0.1% [w/v]); **(b)** Effect of additional nitrogen sources (0.1% [w/v]); **(c)** Combined effect of the addition of glucose and yeast extract (0.1% [w/v] each)

***Additional nitrogen sources***

The nitrogen source also influences the bacterial azo dye decolorization process (Ali 2010; Solís et al. 2012). Therefore, two organic (yeast extract and peptone) and three inorganic (sodium nitrate, potassium nitrate, and ammonium sulfate) nitrogenous compounds were tested for their suitability as an additional nitrogen source for the bacterial consortium when added to the reactive azo dye mixture (50 ppm dye mixture, 10% [w/v] NaCl, pH 10) (Fig. 4b). In the absence of any additional nitrogen source, a color removal of 30.77% was recorded. Compared to the other tested nitrogen sources, yeast extract was found to be the most effective ($p$=0.002), with a 94.99%

color reduction after 72 hours. Similarly, Moosvi et al. (2007) and Jain et al. (2012) reported that yeast extract (0.1 and 0.5% w/v) was an optimal nitrogen source for the decolorization of Reactive Violet 5R by bacterial consortia. Yeast extract metabolism helps in the regeneration of electron carriers such as NADH, which facilitates in azo dye reduction (Chen et al. 2003). Peptone was also found to be an effective nitrogen source when compared to no added nitrogen ($p$=0.004), with 79.79% color removal observed after 120 hours. With the addition of sodium nitrite, potassium nitrate, and ammonium sulfate, a decolorization of 58.63%, 51.72%, and 51.94%, respectively was achieved after 120 hours. The addition of inorganic nitrogenous compounds resulted in lower decolorization percentages than the addition of organic nitrogen sources ($p$=0.003).

***Glucose and yeast extract mixture***

To establish the combined effect of the additional carbon and nitrogen sources on azo dye (50 ppm dye mixture, 10% [w/v] NaCl, pH 10) decolorization by the bacterial consortium, a mixture of glucose and yeast extract (0.1% [w/v] each) was added (Fig. 4c). This resulted in a faster color removal than when glucose ($p$=0.008) and yeast extract ($p$=0.001) were added separately, with 97.19% decolorization achieved in 24 hours compared to 72 hours. The combination of these two compounds synergistically enhanced the color removal process. Similar findings were reported by Lalnunhlimi and Krishnaswamy (2016) in which addition of glucose and yeast extract improved the decolorization of mixed dyes (200 ppm) by an alkaliphilic consortium.

***Temperature***

Temperature is another important factor in dye decolorization as it affects the growth and enzymatic profile of bacteria (Solís et al. 2012). Therefore, decolorization of the reactive azo dye mixture (50 ppm dye mixture, 10% [w/v] NaCl, pH 10, 0.1% [w/v] glucose, 0.1% [w/v] yeast extract) was tested at different temperatures *i.e.* 25, 30, 35, 40 and 45℃ (Fig. 5a). A linear increase in decolorization was observed when temperature was increased from 25 to 35℃. Out of all temperatures tested, the optimal temperature for this bacterial consortium was found to be 35℃ ($p$=0.0001), and 97.59% decolorization was observed after 24 hours. Increasing the temperature past 35$^{o}$C resulted in a decrease in color removal with 64.99% (40$^{o}$C; $p$=0.0004) and 39.95% (45$^{o}$C; $p$=0.01) decolorization observed. Similarly, Arun Prasad et al. (2013) observed a decrease in decolorization percentage at 40 and 45℃, while 37℃ was found to be the optimal temperature for the decolorization of Direct Blue 1 by the moderately halophilic bacterium *Marinobacter* sp. strain HBRA. Contrary to that, Dawkar et al. (2008) observed maximum color removal of Brown 3REL at 40℃ by *Bacillus* sp. VUS. Higher temperatures could be detrimental to cell viability and/or result in the denaturation of enzymes involved in dye decolorization (Pearce et al. 2003; Cetin and Donmez 2006).

***pH***

Effect of pH on the decolorization of the reactive azo dye mixture (50 ppm dye mixture, 10% [w/v] NaCl, 0.1% [w/v] glucose, 0.1% [w/v] yeast extract, 35$^{o}$C) was tested at different pH values ranging from 8 to 13 (Fig. 5b). The optimum pH range was 8 to 11 and a color reduction of 90-98% was observed within 24 hours. The consortium retained its decolorization potential even at high pH values, showing a color removal of 87.08% (pH 12) and 84.89% (pH 13) in 24 hours. This increased to 91.42% (pH 12) and 87.09% (pH 13) with extended incubation (120 hours). To our knowledge, this is the first report of such a high level of pH tolerance for a dye decolorizing bacterial consortium. Bhattacharya et al. (2017) has reported up to 97% decolorization of 50 ppm

Methyl Red by *N. lacusekhoensis* EMLA3 at a pH range of 8-11.5 within 24 hours. However, at higher pH values, *i.e.* 12 and 13, decolorization activity was reduced. In another study, a pH of 9.5 was reported as optimal for the decolorization of 200 ppm Direct Red Blue 151 and Direct 31 by an alkaliphilic consortium within 5 days (Lalnunhlimi and Veenagayathri 2016). The broad range pH tolerance of the present alkaliphilic, halotolerant consortium makes it a promising candidate for treatment of alkaline textile effluents where most of the neutral pH dwelling microbes fail to perform due to disrupted metabolic activities (Amoozegar et al. 2011).

***Initial dye concentration***

Dye mixture decolorization was examined with different initial dye concentrations, *i.e.* 50. 100, 200, 300, 400 and 500 ppm (10% [w/v] NaCl, pH 10, 0.1% [w/v] glucose, 0.1% [w/v] yeast extract, 35$^{o}$C) (Fig. 5c). The alkaliphilic and halotolerant bacterial consortium showed up to 98% decolorization with an initial dye mixture concentration of 50 ppm and 100 ppm within 24 and 72 hours, respectively. With an initial concentration of 200 ppm, up to 89% decolorization was observed in 96 hours. Decolorization activity of the consortium was reduced when the initial dye mixture concentration was increased from 200 to 300 ppm ($p$=0.01), with 66% decolorization achieved after 96 hours. Additionally, the amount of decolorization was substantially diminished at higher dye mixture concentrations when compared to 200 ppm, with only 17% (400 ppm; $p$=0.0001) and 5% (500 ppm; $p$<0.00001) color reduction observed. Similarly, Moosvi et al. (2007) observed a decrease in the decolorization efficiency of the consortium JW-2 for Reactive Violet 5R above the optimal concentration of 100 ppm. Likewise, a decrease in the color removal extent at higher dye concentrations was reported in other studies (Khehra et al. 2005; Jirasripongpun et al. 2007; Joe et al. 2011; Lv et al. 2013). At higher concentrations, textile dyes are usually toxic for bacterial cells, and in the case of reactive dyes, the effect of sulfonic acid groups becomes more pronounced and hinders bacterial growth (Kalyani et al. 2008). Another possible explanation for the reduced decolorization activity is the resulting imbalance in dye to cell ratio. This could lead to blockage of enzyme active sites involved in color removal (Sani and Banerjee 1999; Saratale et al. 2009).

***NaCl concentration***

The salinity of textile effluents can hamper the decolorization activity of non-salt tolerant microbes, as it can lead to cell damage through plasmolysis (Manu and Chaudhari 2003; Aksu 2005; Dafale et al. 2008). Therefore, the ability of the bacterial consortium to decolorize the textile dye mixture was tested with varying NaCl concentrations (50 ppm dye mixture, pH 10, 0.1% [w/v] glucose, 0.1% [w/v] yeast extract, 35$^{o}$C) (Fig. 5d). Considerable color reduction (91-98%) was observed over a range of salt concentrations (0-9%) within 120 hours. With 10% NaCl, 97.89% decolorization was achieved in 24 hours. At higher concentrations, the decolorization percentage was reduced when compared to 10% NaCl (*i.e.* with 12% NaCl, 85.99% color removal was achieved in 120 hours, $p$=0.04). Similarly, Khalid et al. (2012) reported decolorization of different reactive dyes over a range of NaCl concentrations (0-10% [w/v]) with varying color removal rates by *Psychrobacter alimentarius* and *Staphylococcus equorum*. Contrary to that, Arun Prasad et al. (2013) reported optimal decolorization of 100 ppm Direct Blue 1 at 7% (w/v) NaCl by *Marinobacter* sp. strain HBRA.

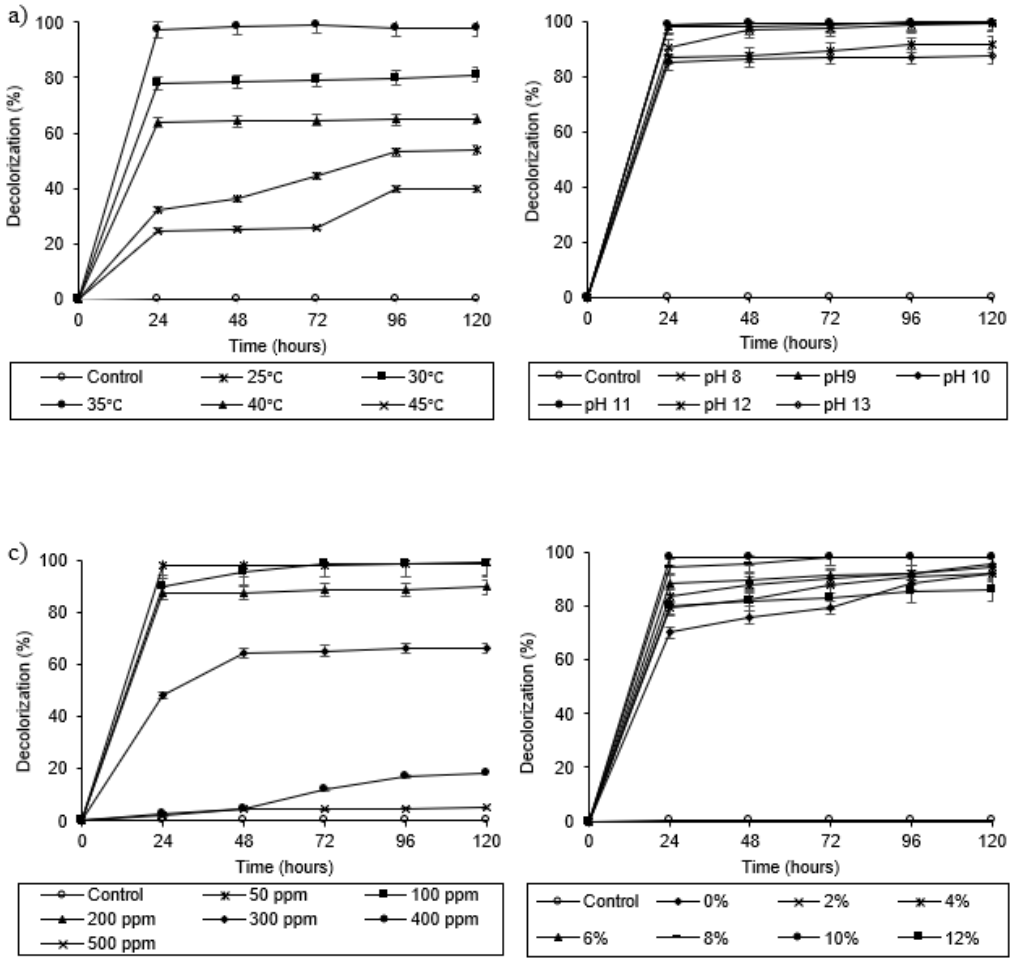



**Fig. 5** Performance of alkaliphilic, halotolerant bacterial consortium for the decolorization of reactive azo dye mixture at different **(a)** temperatures (50 ppm dye mixture, 10% [w/v] NaCl, pH 10, glucose and yeast extract 0.1% [w/v] each); **(b)** initial pH (50 ppm dye mixture, 10% [w/v] NaCl, 35℃, glucose and yeast extract 0.1% [w/v] each ); **(c)** initial dye mixture concentrations (10% [w/v] NaCl, pH 10, 35℃, glucose and yeast extract 0.1% [w/v] each]; and **(d)** NaCl (w/v) concentrations (50 ppm dye mixture, pH 10, 35℃, glucose and yeast extract 0.1% [w/v] each)

**FTIR analysis**

The FTIR spectra (650-4000cm$^{-1}$) of the untreated dye mixture sample (Fig. 6a) and the treated dye mixture sample (Fig. 6b) were measured to determine the difference between the dye and metabolites present. In the untreated dye mixture sample, the band at 3043.99 cm$^{-1}$ corresponded to O–H band vibrations. The band at 2832.68 cm$^{-1}$ corresponded to C–H stretching vibration. N=N stretching was indicated by absorption bands at 1559.75 and 1644.22cm$^{-1}$. The band observed at 1795.57 cm$^{-1}$ corresponded to C=O stretching, while the band at 1439.44 cm$^{-1}$ corresponds to C=N stretching. C–OH stretching was indication by an absorption band at 1243.65 cm$^{-1}$. Band at 1058.16 cm$^{-1}$ corresponds to S=O stretching while the presence of substituted aromatic ring was indicated by band at 992.82 cm$^{-1}$. The band at 726.41 cm$^{-1}$ shows the presence of C–Cl stretch (Lambert et al. 1998; Yuen et al. 2005). Analysis of the treated dye mixture sample showed fluctuations in many bands found in the untreated dye sample (Fig. 6b). Moreover, new peaks were also observed. Most notably, the disappearance of peak at 1559.75 and 1644.22 cm$^{-1}$ indicated –N=N– bond cleavage that resulted in color removal. Variation in band intensity at 2832.68 cm$^{-1}$ indicated C-H

stretching fluctuation. Presence of free N-H groups was indicated by appearance of new bands at 3727.29, 3508.47, and 3408.05 $cm^{-1}$. C=N and S=O bond distortion was indicated by the shift of the bands observed at 1439.44 and 1058.16 $cm^{-1}$, respectively in the untreated dye mixture sample. Deformation of the aromatic ring structure was indicated by the disappearance of the peak at 992.82 $cm^{-1}$ (Lambert et al. 1998; Mohammed and Mohammed 2010; Olukanni et al. 2010).

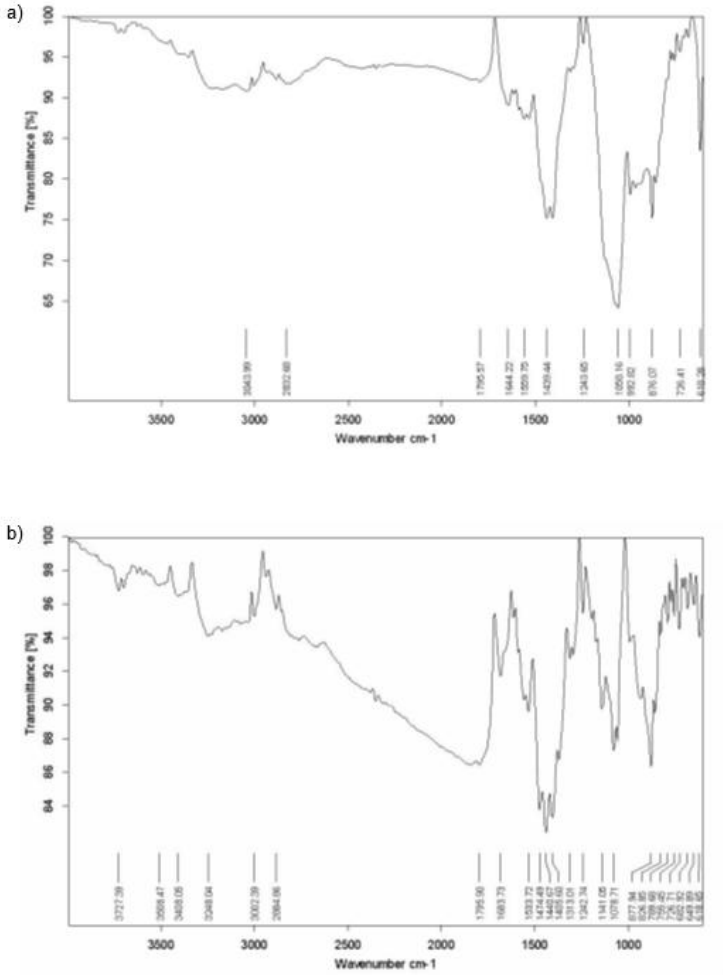



**Fig. 6** FTIR spectra of **(a)** reactive azo dye mixture before bacterial treatment; **(b)** extracted metabolites of reactive azo dye mixture obtained after bacterial treatment

## Toxicity analysis

### *Phytotoxicity assay*

Textile effluents are typically released into open water systems and despite the known threat associated with these waters, they are frequently used for irrigation (Jadhav et al. 2008). Although the harmful effects of textile waste can be reduced through treatment, it is vital to test the toxicity level of the waste following treatment (Kalyani et al. 2008). Therefore, the sensitivity of *Raphanus sativus* seeds towards 50 ppm reactive azo dye mixture (untreated and treated) was tested. Dye free medium was used as a negative control. Seed germination, root length, and shoot length was determined (Table 3). The treated dye mixture sample showed less toxicity when compared to the untreated dye mixture (73.33% and 66.66% germination, respectively) ($p$=0.16). Moreover, the root and shoot length of the seeds watered with the treated dye mixture sample was slightly

higher (1.27 cm [±0.06] and 1.19 cm [±0.21]), respectively) than the root and shoot lengths obtained with the untreated dye mixture sample (1.20 cm [±0.24] and 1.15 cm [±0.53], respectively), though the results were not statistically significant ($p$=0.41 and 0.48 for root length and shoot length, respectively). Likewise, Parshetti et al. (2006) reported reduced toxicity of 50 ppm Malachite Green following bacterial treatment with improved germination, root length, and shoot length of *Triticum aestivum* and *Phaseolus mungo*.

**Table 3.** Phytotoxicity of untreated and treated dye mixture

| Sample | Seed sowed (#) | Seeds germinated (#) | Seed germinated (%) | Mean root length (cm) ± S.E. | Mean shoot length (cm) ± S.E. |
|---|---|---|---|---|---|
| Dye free medium (negative control) | 15 | 13 | 86.66 | 1.86 ± 0.074 | 1.17 ± 0.32 |
| Untreated dye mixture (positive control) | 15 | 10 | 66.66 | 1.20 ± 0.24 | 1.15 ± 0.53 |
| Treated dye mixture | 15 | 11 | 73.33 | 1.27 ± 0.06 | 1.19 ± 0.21 |

S.E. Standard error

***Brine shrimp lethality assay***

Reduction in the toxicity of the 50 ppm reactive azo dye mixture was also indicated by an acute toxicity test, using *Artemia salina* larvae. The mortality of the larvae treated with the decolorized dye mixture sample (30%) was lower than when treated with the untreated dye mixture sample (70%) ($p$=0.05) (Table 4). This is in accordance with, who reported a reduction in toxicity of 750 ppm Methyl Red following decolorization by *Sphingomonas paucimobilis.*

**Table 4.** Cytotoxicity of dye mixture before and after bacterial treatment

| Sample | *A. salina* larvae added (#) | *A. salina* larvae alive (#) | *A. salina* larvae dead (#) | Mortality (%) |
|---|---|---|---|---|
| Dye free medium (negative control) | 10 | 07 | 03 | 30 |
| Untreated dye mixture (positive control) | 10 | 04 | 03 | 70 |
| Treated dye mixture | 10 | 07 | 03 | 30 |

**Conclusion and Future work**

This study confirmed the hypothesis that extremophilic dye decolorizing bacteria could be isolated from a garden soil through acclimation. The ability of acclimated alkaliphilic, halotolerant bacterial consortium to effectively decolorize the azo dye mixture under alkaline pH and high saline conditions, makes it a promising candidate for treatment of such wastewaters. FTIR and toxicity tests indicated the breakdown of these dyes to less toxic products, though further investigations focused on degradation rates and mechanisms are required to gain detailed insight into the treatment process. As this study was conducted under simulated conditions, additional studies to test the potential of this alkaliphilic, halotolerant consortium for the treatment of real textile wastewater would be beneficial. This information will be helpful in the design of pilot and large-scale treatment systems.

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