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EFFECT OF MIXED BACTERIAL CONSORTIA FOR DECOLORIZATION AND DETOXIFICATION OF AN AZO DYE: CI DIRECT BLUE 201 TEXTILE DYE

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ABSTRACT

Synthetic textile dyes generated from textile industry, has become a severe environmental problem due to the xenobiotic properties and toxicity of the dyes. The present study was focussed on the potential application of different bacterial consortium consists with *Alcaligenes faecalis* (MK166784), *Micrococcus luteus* (MK166783) and *Staphylococcus warneri* (MK256311) in A-1 (*A. faecalis*, *M. luteus* and *S. warneri*), A-2 (*A. faecalis* and *M. luteus*), A-3 (*A. faecalis* and *S. warneri*) and A-4 (*M. luteus* and *S. warneri*) consortia for the decolorization of CI Direct Blue 201, sulfonated azo dye. Among the different bacterial consortia tested, the consortium A-2 was found to be the best bacterial consortium for the decolorization of CI Direct Blue 201 dye, than the monocultures of bacteria employed itself. The consortium (A2) achieved complete decolorization of CI Direct Blue 201 textile dye at 48 hrs of incubation under static conditions. Futher, it was found that the bacterial consortium A-2 utilized the dyes efficiently with the supplement of tryptone and peptone as the co-substrates in modified Mineral Salt Medium. Results of the phytotoxicity assay confirmed that the dye was detoxified after the treatment. Thus the treated dye contained water can be used for agrarian purposes.

KEY WORDS : *Alcaligenes faecalis, Micrococcus luteus*, Bioremediation, Consortium, CI Direct Blue 201 textile dye

INTRODUCTION

The public perception of water quality is highly influenced by the color as it is the first contaminant to be recognized in wastewater (Banat *et al.*, 1996). Colored synthetic dyestuffs are extensively used in the textile, paper printing, cosmetic and pharmaceutical industries (Chang *et al.*, 2001 and Moosvi *et al.*, 2007). Synthetic azo dyes, used in textile industries, accounts for two-third of world synthetic dye production (Ekanayake *et al.*, 2017; Gupta, 2009; Jadhav *et al.*, 2008 and Saratale *et al.*, 2009). Textile industries consume a substantial volume of clean and treated water and in turn create a huge load of colored wastewater due to the low level of dye fiber fixation rate (Bumpus, 1995). For instant, direct dyes which used for cotton fibers, losses 10-30% of dyes to the effluent water (Bumpus, 1995 and Cristóvão, 2010). In addition to the color, most synthetic dyes and their biotransformation products have been toxic to the flora and fauna even at low concentrations (Chung *et al.*, 1993 and Robinson *et al.*, 2001). Oral ingestion, inhalation and skin sensitization of textile dyes leads to carcinogenicity (Chung *et al.*, 1993), mutagenicity (Kalyani *et al.*, 2008), chronic toxicity (Reemtsma *et al.*, 2006), respiratory diseases (Chung *et al.*, 1993), skin irritation (Chung *et al.*, 1993), biodiversity collapse of aquatic fauna (Gupta, 2009), flora (Vandevivere *et al.*, 1998) and human health (Chequre *et al.*, 2011). Changes of water and soil pH, COD, BOD, conductivity, DO and low light penetration level of water has received greater attention due to release of textile wastewater to the

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aquatic systems (Kalyani *et al.,* 2008).

Dyes are designed to be resistant to natural degradation processes, thus persist in the environment for a long time (Chu, 2001 and Ekanayake and Manage, 2016). Todate, different physical (Robinson *et al.,* 2001), chemical (Banat *et al.,* 1996) and biological treatment methods (Ekanayake *et al.,* 2017; Jadhav *et al.,*2008 and Saratale *et al.,* 2009) are being practiced all over the world. However, most of the physical and chemical methods are not applicable in all part of the world due to the high initial and maintenance cost with formation of a heavy load of sludge (Konsowa, 2003 and Robinson *et al.,* 2001). Thus, biological treatment methods are one of the emerging applications for treatment of synthetic dyes as an eco-friendly and economical alternative without creating secondary pollutants to the environment.

Biological agents; bacteria (Ekanayake *et al.,* 2017, Kalyani *et al.,* 2008 and Moosvi *et al.,* 2007), fungi (Bankole *et al.,* 2018 and Ekanayake *et al.,* 2018), cyanobacteria (Anandhana *et al.,* 2018) and aquatic plant (Ekanayake *et al.,* 2016 and Kagalkar *et al.,* 2009) have been recorded as potential candidates for decolorization and detoxification of various structurally different textile dyes. Considering the long growth cycles of fungi, decolorization and detoxification rate of textile dyes by bacteria is generally fast (Banat *et al.,* 1996). Recent studies have reveled that the bacterium *Micrococcus* sp. (Ekanayake *et al.,* 2017), *Bacillus* sp. (Ooi *et al.,* 2007), *Pseudomonas* sp. (Kalyani *et al.,* 2008) are potential microbial candidates for the decolorization of wide range of textile dyes. However, published information recorded that most of the individual bacterial strain along, cannot degrade azo dyes completely and the resulted intermediate products are need to be further decomposed using another biological agent (Haug *et al.,* 1991). Hence, use of bacterial consortium concept has been suggested as the most appropriate microbial method to remove textile dyes as different metabolic activities and syntrophic interaction of individual bacteria in a bacterial consortium leads to the complete mineralization of dye structures (Chang *et al.,* 2004; Ekanayake *et al.,* 2019a; Fernando *et al.,* 2012; Nigam *et al.,* 1996 and Saratale *et al.,* 2009). In Sri Lankan contest, treatment of textile dye effluents is done by chemical methods where the discharge wastewater effluent is significantly contribute to water and soil pollution (Mahagamage *et al.,* 2014 and 2015). Thus, an effective microbial treatment methods, rather than chemical treatment is timely needed to protect both ground and surface water. Therefore, the present study was aimed to evaluate the role of bacterial consortium for decolorization of an azo dye; CI Direct Blue 201 (DB 201) as microbiological treatment method.

MATERIALS AND METHODS

Synthetic dyes and chemicals

DB 201 (834 g mol^{-1}), a di-azo direct dye, was selected as the model dye for the study based on the usage of the dye in Sri Lankan textile industry. All the other chemicals were in microbiology and analytical grade with the highest purity.

Culture media

Luria Bertani (LB) medium (g/L: 4.6 of yeast extract and 15 of tryptone water) (Chang *et al.,* 2001) was selected as the general growth medium for bacteria. Around 1.5% bacteriological agar was added when solid medium was required. Liquid medium screening of textile dye decolorization was monitored in modified Minimal Salt Medium (MSM) (g/L: 3.39 Na_2HPO_4, 5.0 NH_4Cl, 15.0 KH_2PO_4, 3.5 $MgCl_2$, 2.5 NaCl) (Asad *et al.,* 2007). The pH of the medium was adjusted to 7.0 unless otherwise stated.

Bacteria and culture conditions

Three bacterial strains; *Alcaligenes faecalis* (MK166784), *Micrococcus luteus* (MK166783) and *Staphylococcus warneri* (MK256311) which were previously isolated and identified as the textile dye decolorizing agents, by the authors (Ekanayake *et al.,* 2019b), were used in the present study. Pure cultures were prepared and maintained on LB Agar slants which was amended with 5% DB 201 textile dye and stored at 4 ^{o}C, subjected to sub culture once a month.

Dye decolorization studies in the liquid medium

The DB 201 textile dye decolorization potential of individual bacterial strains and the mixed bacterial consortia were evaluated. Each bacterial strain was grown overnight in LB medium, centrifuged 8000 rpm for 15 minutes and washed with 0.9% saline (Idroos *et al.,* 2018; Manage *et al.,* 2009 and 2010) and the washing procedure was followed for three times to ensure all the carbon sources in the growth medium were removed. Thereafter, the bacterial

cells were starved overnight in 0.9% saline (Idroos *et al.*, 2018) and turbidity was equalized to 0.350 at the wavelength of 595 nm using UV-Vis spectrophotometer to maintain more or less similar amount of bacterial cells (1-2 x 10^8 cells/mL) at each step in the experiment (Manage *et al.*, 2000 and 2010). For the monocultures, 5 mL of the equalized bacterial suspension was introduced into 100 mL of modified MSM at 50 mg/L of final concentration of DB 201 textile dye. The treated samples were incubated at 28±1 ^{0}C for 14 days, under static condition. Three milliliter of aliquot was removed 24 hrs, centrifuged and absorption was measured at 570 nm using the UV-VIS spectrophotometer (Ekanayake *et al.*, 2017). The effciency of the employed bacterial consortium for the decolorization of CI Direct Blue 201 textile dye was evaluated following the same procedure described. Table 1 illustrates the reference numbers for the each bacterial consortia and the amount of the equalized bacterial volume used.

All the experiments were carried out in triplicates and controls were maintained without addition of bacteria. Decolorization percentage was calculated following the equation given bellow (Gupta *et al.*, 2016), where C1 is initial concentration and C2 is the final concentration of the dye.

Decolorization percentage (%) = [(C1-C2)/C1]100

Optimization of the bacterial consortium for decolorization of DB 201 textile dye

The effect of different operational and environmental conditions on the decolorization of DB 201 textile dye against the bacterial consortia was studied. The effect of different temperature (24, 28, 32, 36 and 40 ^{0}C), pH (5, 6, 7, 8, 9 and 10), static or shaking conditions (50 and 100 rpm), initial dye concentration (25, 50, 75, 100 and 150 mg/L) and co-substrates (starch, glucose, yeast, tryptone, peptone, urea) were evaluated by changing each parameter at once, while keeping the others constant whereas controls were maintained without addition of bacteria. All the experiments were carried out in triplicates and incubated the same period of time at 28 °C, pH 7, under static conditions, unless otherwise stated. Decolorization was measured by following the equations given in 2.4. Consecutive addition of dyes was evaluated by adding 50 mg/L of DB 201 textile dye at the end of each decolorization cycle, without further supplement of nutrients into the system.

Phytotoxicity assay

The toxicity of the original dye and the decolorized dye solutions by the bacterial consortia were evaluated by the phytotoxicity assay. *Oryza sativa* (monocot) and *Vigna radiate* (dicot) seeds germination assay was used to evaluate the toxicity (Ekanayake *et al.*, 2019 and Kalyani *et al.*, 2008). In this experiment, 30 seeds of each plant species were placed on moisture chambers having several layers of paper tissues on petri dishes. Seeds were watered (5 mL) by DB 201 textile dye (50 mg/L) and decolorized dye solutions, separately. Modified MSM without addition of dye was used as the control setup and seed germination percentage of each set up was calculated in three days of incubation. All the experiments were carried out in triplicates.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the data, and results were considered as significant when $p < 0.05$.

RESULTS AND DISCUSSION

Comparative study of the decolorization of DB 201 textile dye by individual bacterial strains and bacterial consortia

Decolorization potential of individual bacterial strains (*A. faecalis*, *M. luteus*, *S. warneri*) and the bacteria consortia (A-1, A-2, A-3, and A-4) were screened for different environmental and operational conditions (Fig. 1). The time taken to reach complete decolorization was varying

Table 1. Bacterial strains and equalized (0.350) bacterial volumes used to prepare the bacterial consortium

Bacterial consortium	Representative bacteria in each consortium	Volume of equalized bacteria at 0.350 in 100 mL broth
A-1	*A. faecalis, M. luteus, S. warneri*	1.66 mL each
A-2	*A. faecalis, M. luteus*	2.5 mL each
A-3	*A. faecalis, S. warneri*	2.5 mL each
A-4	*M. luteus, S. warneri*	2.5 mL each

according to individual bacterial strains and different bacterial consortia even at the same environmental conditions provided. The bacterial consortium: A-2 (*A. faecalis* and *M. luteus*) showed the greatest dye decolorization potential reaching complete decolorization (100%) of the DB 201 dye at two days of incubation, where other individual bacteria and consortia showed less than 80% of decolorization at the same incubation time and conditions. Banat *et al.* (1996) showed that the textile dye decolorization behavior of different bacteria species may vary with the conditions required for their growth. Further, a study done by Chen *et al.* (2007) stated that the decolorization of textile dyes may enhanced due to the synergistic reaction of bacteria strains in the consortium or vice versa.

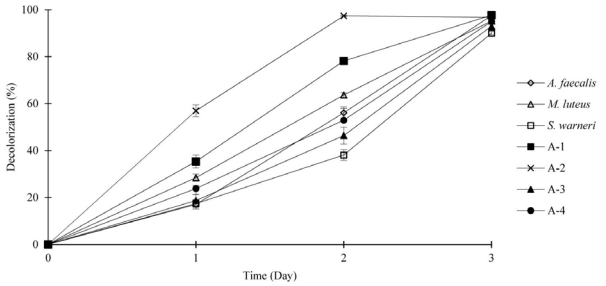

Fig. 1. Decolorization of DB 201 textile dye by individual and bacterial consortia (when error bars are not shown, standard deviations are less than the width of the symbol).

The selected textile dye for the present study (DB 201), has sulfur group, two azo bonds and higher molecular weight (834 g mol^{-1}). Moosvi *et al.* (2005) recorded that, azo dyes with sulphur groups and higher molecular weights are the factors that slower bacterial growth as well as the dye degradation rate. Interestingly, in the present study, the bacterial consortium; A-2 was able to show complete decolorization of DB 201 dye within 48 hrs. Remarkably, few researchers have recorded the significant decolorization of some selected textile dyes when dyes exposed to the bacterial consortium than the individual bacteria, suggesting that individual strains may attack the dye molecules at different positions or may utilize the metabolized produced by the co-existing strains for further decomposition (Joshi *et al.*, 2008; Moosvi *et al.*, 2007, Saratale *et al.*, 2009 and 2010). Further, Saratale *et al.* (2010) explained that the effectiveness of microbial decolorization of textile dyes depend on the survival, adaptability and the enzymatic activities of the microorganisms present in the consortium. Therefore, the decolorization pathways of the *A. faecalis* and *M. luteus* employed in the present study may be matching each other which enhance growth of the bacterium leading to the decolorization of the dye, efficiently. The reduction of the decolorization of the DB 201 textile dye by the other consortia may due to the interferences occurs among the bacteria or dismatch of their co-existing in the given environment. Some studies have highlighted that the textile dye decolorization processes by biological agents highly depend on the type of the textile dyes used and the species employed. For instance, Joshi *et al.* (2008) recorded the 90% decolorization of Acid Orange 7 dye within 16 hrs when the bacteria *Aeromonas caviae*, *Proteus mirabilis* and *Rhodococcus globerulus* employed as a consortium where Nigam *et al.* (1996) recorded the complete decolorization of Cibacron Red, Remazol Golden Yellow, and Remazol Red within 24-30 hrs under anaerobic conditions by the mixed bacterial consortium consists with *A. faecalis* and *Comamonas acidovorans*. Further, Moosvi *et al.* (2007) recorded complete decolorization of Reactive Violet 5R dye within 36 hrs by the bacteria consortium consist of *Paenibacillus polymyxa*, *M. luteus* and *Micrococcus* sp. while Saratale *et al.* (2009) showed complete decolorization of Scarlet R dye and Green HE4BD dye by the mixed bacterial consortium of *Proteus vulgaris* and *Micrococcus glutamicus*. In the present study, the bacterial consortium: A-2 showed the highest decolorization potential for DB 201 textile dye within 48 hrs. Thus, the bacterial consortium A-2, was selected for further studies to evaluate the effect of bacterial consortia on decolorization of DB 201 dye.

Optimization of decolorization of DB 201 textile dye for selected physico-chemical conditions

Temperature and pH in the textile wastewater are highly depended on the type of dye used and the process of the application of dyes to the fibre. Thus, it is mandatory to evaluate the effect of different environmental and operational parameters on bacterial dye decolorization processes. BOI. (2010) guideline given for the discharge of textile wastewater effluent in Sri Lanka, temperature of the effluent water should below the 40 °C. Accordingly, the effect of temperature on decolorization of DB 201 textile dye was evaluated at 24, 28, 32, 36 and 40 °C respectively and it was found that the dye decolorization was not significantly different at the temperature range from 28 °C to 40 °C and only 24

°C showed reduction of the decolorization (84%) (Fig. 2A). Control experiment setup showed less than 1% dye decolorization for all temperatures tested.

Effect of pH by the decolorization medium is highly influence on the growth of bacteria and their enzymatic activities (Lourenco *et al.,* 2000 and Saratale *et al.,* 2010). The color of a dye is highly depended on pH of the dissolving solvent. Thus, the initial color of the dye was recorded prior to the decolorization experiments, and no deviations of the maximum absorption spectra were found at the pH range of 5-10. In the present study, decolorization was assessed under different pH (5, 6, 7, 8, 9 and 10). However, with the application of bacteria consortium, the highest dye decolorization was recorded at pH 7 (100% at 48 hrs of incubation) and dye decolorization percentages were lower at pH 5 (54%) and 10 (56%) at the same incubation time (Fig. 2B). More or less similar results were recorded by Moosvi *et al.* (2007) where the most effective pH for Reactive Violet 5R dye decolorization by the consortium of *P. polymyxa, M. luteus* and *Micrococcus* sp. was within the range of pH 6.5 to 8.0.

The preference of static or shaking conditions for decolorization of textile dyes is highly depended on the bacteria employed. The complete decolorization of DB 201 dye by the bacterial consortium A-2 was achieved within 48 hrs under static conditions while 51% (50 rpm) and 28% (100 rpm) of dye decolorization percentages were recorded at the shaking condition (Fig. 2C). In the control set-up, less than 2% of decolorization was recorded under the shaking condition. Saratale *et al.* (2010) recorded 30% decolorization for Green HE4BD dye when the bacterial consortium applied at shaking condition where complete decolorization was observed under static conditions. A study conducted by Moosvi *et al.* (2007) presented a similar dye decolorization pattern for RV5R dye when the dye exposed to a bacterial consortium. Further, Saratale *et al.* (2010) suggested that the preference of facultative anaerobic conditions by bacteria enhance the rapid decolorization of textile dyes and this could be due to the involvement of azoreductase like different type of enzymes. Chang *et al.* (2001) reported that the reductive cleavage activity of azo bond by azo reductase enzymes which are normally inhibited with the presence of oxygen, due to the competition of oxidation of reduced electron carriers like NADH with either oxygen or azo groups as electron

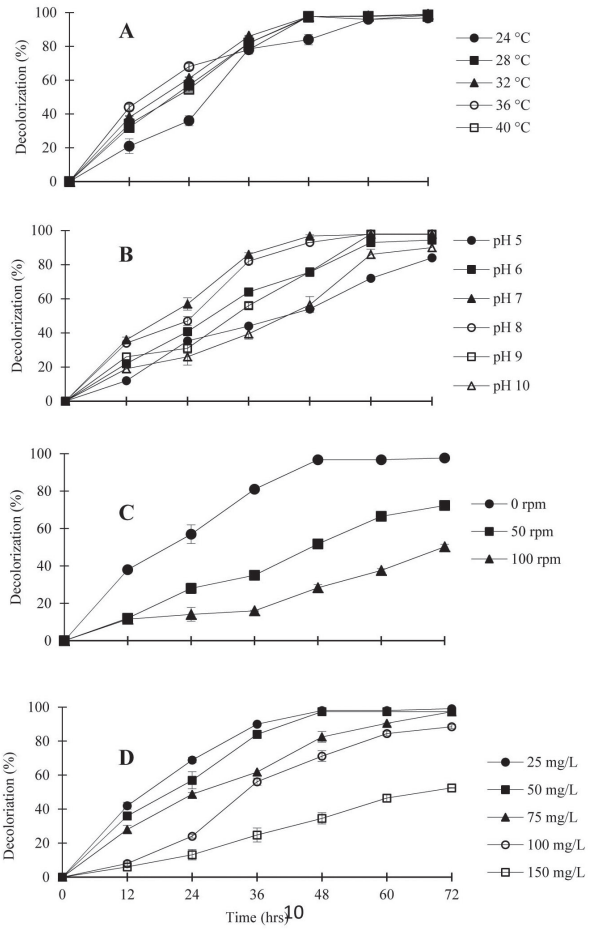

Fig. 2. Effect of different conditions for decolorization of DB 201 dye by A-2 bacterial consortium A: temperature, B: pH, C: shaking, D: initial dye concentration (when error bars are not shown, standard deviations are less than the width of the symbol). Control lines are not shown when the results were less than 2 %.

receptors. Accordingly, decolorization of DB 201 textile dye by the bacterial consortium employed in the present study indicates the involvement of some enzymatic reactions of the bacteria against dye in the medium.

Complete decolorization of dye was observed within 48 hrs of incubation, for 25 and 50 mg/L of initial DB 201 dye concentrations (Fig. 2D). The consortium A-2 obtained 72 hrs to reach complete dye decolorization when the initial dye concentration was increased into 75 mg/L. Afterword, descending pattern of dye decolorization was observed as a response to the increasing initial dye concentrations. The control experimental set-up showed less than 2% of dye decolorization. Moosvi *et al.* (2007) and Saratale *et al.* (2010) have recorded

gradual decrease of the decolorization of some azo dyes by different bacterial consortia suggesting that the reduction of decolorization at high initial dye concentrations is due to the toxicity effect of the dye on bacterial biomass or blockage of the active sites of the enzymes by dye molecules. Hu (1994) also reported that the presence of sulphonic groups reduced the decolorization potential of bacterial strains.

Performance of the A-2 bacterial consortium in decolorization of DB 201 textile dye was evaluated by amending the MSM with different co-substrates which are rich with Carbon and Nitrogen. It was found that the presence of tryptone and peptone in the MSM enhanced the decolorization of dye by the bacterial consortium A-2 (Fig. 3). However, supplement of starch into the MSM did not show any significant effect on the decolorization of the dye where the control set-up employed with different co-substrates showed less than 3% of decolorization. Saratale *et al.* (2009) recorded that decolorization of Scarlet R dye by the bacterial consortium consists of *P. vulgaris* and *M. glutamicus*, was not effective in the synthetic medium without supplement of additional carbon sources as textile dyes are deficient with freely available carbon and nitrogen sources. Thus, biodegradation of textile dyes is fairly difficult without supplement of extra carbon and nitrogen sources (Moosvi *et al.*, 2007 and Senan *et al.*, 2004). The limited researches have been successful to isolate bacteria capable for utilizing textile dyes as their sole carbon source (Ekanayake *et al.*, 2017 and Moosvi *et al.*, 2007). The previous studies have showed that the effect of co-substrates on the decolorization of synthetic dyes may depend on the bacteria employed and type of the dye used (Moosvi *et al.*, 2007 and Senan *et al.*, 2004). Saratale

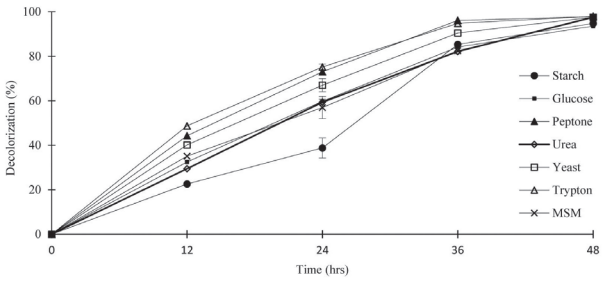

Fig. 3. Effect of differnet co-substrates on the decolorization of DB 201 dye by A-2 bacterial consortium (when error bars are not shown, standard deviations are less than the width of the symbol). Control lines are not shown when the results were less than 3 %.

et al. (2009) recorded the decolorization of Scarlet R dye by the bacterial consortium; *P. vulgaris* and *M. glutamicus*, enhanced with the presence of peptone and beef extract where Moosvi *et al.* (2007) recorded that the enhance of the decolorization of RV5R dye by the bacterial consortium of *P. polymyxa*, *M. luteus* and *Micrococcus sp*. with presence of glucose and yeast in the medium. The presence of different carbon and nitrogen sources in the medium enhance the decolorization ability due to the changes of enzymatic effect in the system (Jadhav *et al.*, 2008). The presence of glucose in the medium which enhances the production of NADH, FADH like reduced necleotides lead to boosts the decolorization efficiency by means of enzymatic activities (Jadav *et al.*, 2008 and Khehra *et al.*, 2005). Organic nitrogen can regenerate the NADH, which acts as electron donor in enzymatic activities (Hu, 1994) where the presence of carbon source enhance the cell growth of bacteria was documented by Moosvi *et al.* (2007).

The repeated usability of the bacterial consortium in decolorization of DB 201 textile dye was evaluated by adding 50 mg/L dye at final concentration at the end of each decolorization cycle, without addition of nutrients. Complete decolorization was observed up to 3 cycles and then followed a descending pattern while control was remained as it is without any color change (Table 2). The reduction of the dye decolorization potential of the bacterial consortium may due to the depletion of the nutrients in the medium or exhaustion of the bacteria as a result of the toxicity of the dye (Saratale *et al.*, 2009).

Table 2. Decolorization of DB 201 dye at consecutive addition of dyes

Decolorization cycle	Decolorization (%)
1st cycle	CD (48 hrs)
2nd cycle	CD (30 hrs)
3rd cycle	CD (30 hrs)
4th cycle	98 ± 1.6 (48 hrs)
5th cycle	82 ± 2.6 (48hrs)

CD: Complete Decolorization (100%)

Phytotoxicity assay

CI Direct Blue 201 is a sulfonated aromatic azo dye. Such azo dyes and most of their metabolic intermediates have properties which can be toxic to the receiving environments (Kalyani *et al.*, 2009). However, most textile wastewaters can be used for agricultural purposes as textile industries produce

Table 3. Phytotoxicity assay for DB 201 and decolorized byproduct by bacterial consortium A-2

Examined parameter	*O. sativa*			*V. radiate*		
	Control	Dye	Decolorized byproduct by A-2	Control	Dye	Decolorized byproduct by A-2
Germination (%)	100	9.0 ± 1.0	100	100	5.0 ± 2.6	100
Plumule (cm)	2.4 ± 0.3	0.9 ± 0.2	2.4 ± 0.1	1.6 ± 0.2	0.5 ± 0.3	1.4 ± 0.2
Radical (cm)	1.6 ± 0.3	0.4 ± 0.2	1.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.5 ± 0.1

substantial amount of water as effluent.

Therefore, toxicity of the original dye and its decolorized solution by the bacterial consortium A-2 were evaluated by phytotoxicity assay. The results showed, 100% germination of *O. sativa* and *V. radiate* after three days in decolorized dye treated plates, where only 9% and 5% germination were observed for *O. sativa* and *V. radiate*, respectively in the plates treated with the original dye (Table 3). Foregoing results indicated that decolorized dye solutions are not toxic for the plant germination and growth emphasizing the practical applicability of the treated dye solutions for agrarian purposes.

CONCLUSION

The potential applicability of the isolated bacterial strains: *A. faecalis* (MK166784), *M. luteus* (MK166783) and *S. warneri* (MK256311) as monocultures and consortia, for the decolorization of CI Direct Blue 201 textile dye was evaluated invitro. The bacterial consortium; A-2, comprising of *A. faecalis* and *M. luteus*, was identified as the best co-existers for decolorization of CI Direct Blue 201 textile dye. The bacterial consortium; A-2 showed rapid dye decolorization at range of pH 6-9, under static condition with the presence of tryptone and peptone as carbon and nitrogen sources. The bacterial consortium (A-2) was able to complete decolorization and detoxification of DB 201 textile dye emphasizing the potential utilization of the treated water for agrarian purposes. Further studies are required to elucidate the textile dye decolorization mechanism by the bacterial consortium to validate the isolates as promising co-candidates for dye bioremediation.

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