Green Approach for Decolorization and Detoxification of Textile Dye- CI Direct Blue 201 Using Native Bacterial Strains

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ABSTRACT

One hundred and fifty six native bacterial strains with different morphological characters were isolated from water and soil samples collected from textile wastewater effluent sites, Sri Lanka. Three isolated bacterial strains were more effective on decolorization of CI Direct Blue 201 textile dye and 16s rRNA analysis reveals that the bacterial strains were *Alcaligenes faecalis* (MK166784), *Micrococcus luteus* (MK166783) and *Staphylococcus warneri* (MK256311). *A. faecalis, M. luteus* and *S. warneri* showed complete decolorization of CI Direct Blue 201 textile dye within 60, 64, and 72 h of incubation time respectively under the static conditions at 28 ºC. Decolorization was effective at a temperature range from 24 ºC to 40 ºC and pH range from 7 to 9. The presence of tryptone, peptone or yeast in the Mineral Salt Medium enhanced the decolorization of the dye. Phytotoxicity assay based on the seed germination percentages of *Oryza sativa* and *Vigna radiate* showed that the detoxification of CI Direct Blue 201 textile dye after the bacterial treatment was effective signifying the potential applicability of the *A. faecalis, M. luteus* and *S. warneri* to develop a green application to treat textile wastewater.

1. INTRODUCTION

The natural coloring materials which derived from plant, animal or mineral sources have played an important role in every civilization, until the first synthetic dye: "mauveine" was discovered in the late nineteenth century (Hunger, 2003). However, synthetic dyes have invaded the textile dyeing industry rapidly due to the excellent color fastness ability, lower production cost, broad range of color spectra, etc. (Bechtold and Mussak, 2009). At present, more than 7×10^5 kg of synthetic dyes are consumed annually in the forms of 100,000 structurally different colors to fulfill the requirement of rapidly changing fashion trends (Gupta et al., 2012).

Textile dyeing industry consumes a huge quantity of water in their manufacturing processes including dyeing, washing, and seizing etc. Around 10-20 L of water is required for dyeing and finishing 1 kg of fabric (Naresh et al., 2013). Annually, more than one thousand tons of dyes together with different types of flame retardants, aromatic amines, bisphenols, heavy metals, etc. are discharged directly either into waterways or to the environment (Rovira and Domingo, 2018). Therefore, textile wastewater effluent interrupts the wellbeing of aquatic ecosystems by deteriorating the quality of surface and groundwater (Ileperuma, 2000; Mahagamage and Manage, 2014; Mahagamage et al., 2015). Finally, the discharges trigger the formation of carcinogenic, microtoxic, mutagenic diseases in terrestrial animals as well as in human beings (Gupta et al., 2009).

Although there are various important control measures for textile wastewater effluents, treatment of textile wastewater is difficult due to the huge daily load of wastewater, high recalcitrance for natural degradation processes, presence of sulfur like additives, high COD levels, etc. (Saratale et al., 2009). Most of the color removal methods work either by concentrating color molecules into sludge or complete degradation of color molecules by breaking the complex structures into colorless daughter products by chemical treatment (Pearce et al., 2003). Most physical and chemical based treatment methods such as electrokinetic coagulation, membrane filtration, activated carbon or peat concentrate the colored effluent by forming a huge amount of sludge

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(Konsowa, 2003; Robinson et al., 2001). Therefore, such application methods create secondary pollution and require millions of dollars for the treatment process to remove the sludge which is highly toxic and recalcitrant for natural degradation processes and has become one of the major problems in terms of point source environmental pollution. Thus, such methods are not applicable all over the world. Countries like Sri Lanka, India, and Bangladesh which are the leading suppliers of apparel to the world, are facing environmental pollution problems with huge use of textile dyes in the textile industries and are compelled to explore alternative novel treatment methods to achieve the required wastewater consent limits and for further expansion of their productions.

Biological treatment of textile wastewater is one of the emerging techniques as an eco-friendly alternative for existing physiochemical treatments. Decolorization by biological agents involved in the biotransformation of toxic textile dyes to non-toxic secondary metabolites with a very little amount of sludge (Kalyani et al., 2008; Saratale et al., 2009). Biological agents including bacteria (Ekanayake and Manage, 2017; Kalyani et al., 2008), fungi (Ekanayake et al., 2018), aquatic plants (Ekanayake and Manage, 2016) and algae (Anandhana et al., 2018) have been reported by a number of investigators with reference to decolorization of various textile dyes to date.

In Sri Lanka, Somasiri et al. (2005) and Wijetunga et al. (2007) have recorded the decolorization of a few acid dyes by anaerobic mixed cultures. According to the author's knowledge, only a limited number of studies are available in Sri Lanka regarding decolorization of di-azo direct dyes (Ekanayake and Manage, 2017), which is a class of dye having the highest rate of toxicity among all the synthetic dye classes (Shore, 1996). Therefore, the present study was designed to isolate, identify and optimize the CI Direct Blue 201 (DB), a di-azo direct textile dye, decolorizing native bacteria as per the request of local textile dyeing industries to achieve the greener certificate of textile wastewater treatment focusing Sri Lankan textile industry into "Product without Guilt Concept".

2. METHODOLOGY

2.1 Synthetic dye and chemicals

A questionnaire survey was conducted to select major dyes used in Sri Lankan textile dyeing industry and based on the results, CI Direct Blue 201 (DB), synthetic azo dye, was selected as the model dye for the study. The chemical structure of the DB textile dye consists of two azo bonds, three sulphate groups on seven aromatic rings having a molecular weight of 834 g/mol (Chemical Book, 2017). The selected dye is generally bound into cotton and other cellulosic materials by electrostatic forces when the aqueous dye bath consists of salts and electrolyte (Hunger, 2003). The DB synthetic azo dye was provided by local textile industry, Poogoda, Sri Lanka. All the culture media, organic and inorganic compounds are in microbiology and analytical grade with the highest purity, obtained from Sigma Aldrich.

2.2 Medium

Luria Bertani (LB) medium consisted of (g/L) Yeast extract (4.6), tryptone water (15) was selected as the general growth medium and modified Mineral Salt Medium (MSM) with (g/L) 3.39 Na_2HPO_4, 15.0 KH_2PO_4, 5.0 NH_4Cl, 2.5 NaCl, 3.5 $MgCl_2$ (Asad et al., 2007) was selected for the decolorization experiment. 1.5% bacteriological agar was added when solid medium was required. The pH of the medium was adjusted to 7.0.

2.3 Isolation and screening of textile dye decolorizing bacteria

The wastewater and soil samples collected from textile wastewater effluent sites in Awissawella (6º58'58.02" N, 80º07'23.86" E) and Biyagama (6º57'28.85" N, 79º59'41.21" E) were subjected to enrichment studies for 14 days at 28±1 ºC under static conditions, spiking of 50 mg/L of DB dye as the sole carbon source. Exactly 1 mL of enriched samples were subjected to serial dilution and plated on LB agar to isolate bacterial colonies with different morphological features and the isolated bacterial strains were maintained in LB slants at 4 ºC (Liyanage and Manage, 2016).

2.4 Textile dye decolorization experiments

2.4.1 Dye assay and dye decolorization by isolated bacterial strains

Decolorization of textile dye could be seen visually compare to the control setup, and determination was done numerically by measuring the absorption using UV-Vis spectrophotometer at relevant wavelength. The pH of DB dye was found to be 8.1, thus, the pH of the culture medium was adjusted to 7.0. Accordingly, the maximum absorption spectra were recorded for pH 6, 7, 8, and 9 in order to ensure the effect of pH changes on color spectrum

of DB dye and all the spectra were found as the same at λ max of 570 nm. The decolorization percentage of the dye was calculated using the equation given below (Gupta et al., 2012), where C1 is the initial concentration and C2 is the final concentration.

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

Overnight exponentially grown bacterial cultures were washed with 0.9% saline solution following centrifugation and starved overnight (Idroos and Manage, 2018) to remove all the remaining carbon sources in the medium and then the turbidity of the each bacterial suspension was equalized to 0.350 at the wave length of 590 nm using a UV-Vis spectrophotometer (Manage et al., 2000; Manage et al., 2009). The equalized bacterial suspension (5% v/v) was introduced into modified MSM spiked with filter sterilized dye (final concentration of 50 mg/L) as the sole carbon source and further incubated at 28±1 ºC for a period of 14 days under static condition. Triplicate sample setup was maintained for each bacterial strain while controls were maintained under the same conditions without the addition of bacteria. The primary screening was carried out for all the bacterial strains isolated in the present study. The changes in absorbance were measured at 14 days of incubation, and the decolorization percentages were calculated accordingly. Any bacterial strain which showed over 80% of decolorization was selected for further studies.

In secondary screening, decolorization was further analyzed by subsampling of 3 mL of aliquots at twenty-four hours' intervals. The most promising bacterial strains showing decolorization of DB were screened and identification was done by 16S rRNA analysis via the service obtained from GENETECH (PVT), Sri Lanka. The 16S rRNA sequences of selected bacterial strains were aligned to the most similar sequences in the National Center for Biotechnology Information (NCBI) using the align sequence program (NCBI, 2018). The pre-aligned 16S rRNA sequences were deposited in GenBank (NCBI, 2018) and accession numbers were obtained.

2.4.2 Optimization of bacterial decolorization of DB textile dye

Decolorization of DB textile dye by the selected isolated bacterial strains were optimized by altering the temperature (24, 28, 32, 36, 40 ºC), pH (6, 7, 8, 9), carbon sources (starch, glucose, yeast), nitrogen sources (tryptone, peptone, urea) and static or shaking conditions (100 rpm). Decolorization experiments were carried out by changing one parameter at once, in the modified MSM at 50 mg/L of final dye concentration following the procedure described in 2.4.1. Moreover, the decolorization potential of selected bacteria strains for different dye concentrations (25, 50, 75, and 100 mg/L) was studied. Repeated usability of selected bacterial strains in decolorization process was studied by addition of 50 mg/L DB textile dye at the end of each decolorization cycle without supplement of nutrients further. All the experiments were carried out in triplicates and controls were maintained for each experiment set up under the same experimental conditions without addition of bacteria.

2.5 Phytotoxicity study

Phytotoxicity assay was employed for the evaluation of toxicity of the original dye and the end product of the bacterial decolorized dye solutions from each bacterial strain employed by the method described by Kalyani et al. (2008) with minor modifications. Thirty seeds of *Oryza sativa* (monocot) and *Vigna radiate* (dicot) were placed on moisture chambers having several layers of tissues on petri dishes and seeds were watered (5 mL) from DB textile dye (50 mg/L) and decolorized dye solutions for each bacterial decolorized dye solutions separately. Modified MSM without addition of dye was used for the control set. Seed germination percentage was calculated at five days of incubation.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of DB textile dye decolorizing bacteria

A questionnaire survey was conducted among Sri Lankan textile dyeing industries to select a model dye for the study. Based on the results of the survey, DB dye was selected for the present study. The selected DB dye is widely used in small scale textile dyeing industries (5-50 kg/day) where the proper treatment methods are not practiced due to the high cost.

Ekanayake and Manage (2016) recorded that DB, is highly recalcitrant to natural photolysis and removal was suggested via biodegradation processes. However, it has been recorded that dyes having azo and sulfo groups on the aromatic components are highly inhibiting the breakdown through bacterial degradation processes (Saratale et al., 2009).

In the present study, 156 bacterial strains having different morphological characteristics were isolated from water and soil samples collected from textile wastewater effluent sites in Sri Lanka and their decolorization capability of DB textile dye was evaluated. The selected bacterial strains were identified using the 16S rRNA analysis (Table 1) and the nucleotide sequences were deposited on the GenBank and accessions were obtained as *Alcaligenes faecalis* (MK166784), *Micrococcus luteus* (MK166783), and *Staphylococcus warneri* (MK256311) respectively.

Table 1. Identification of isolated bacterial strains

Isolate	Number base pair of sequence	Number base pair of homology	Similarity percentage (%)	GenBank accession number
Alcaligenes faecalis	1031	940	99	MK166784
Micrococcus luteus	1018	945	99	MK166783
Staphylococcus warneri	1003	945	99	MK256311

3.2 Optimization of the decolorization under selected parameters

A. faecalis, M. luteus, and *S. warneri* showed complete decolorization of the DB dye within 60, 64 and 72 h of incubation under static conditions, respectively (Table 2). Hassan et al. (2013) have recorded that 70% decolorization of Novacron Orange by the bacterium *M. luteus* within 72 h of incubation suggesting that the dye decolorization potential of bacteria vary with the type of the textile dye. Further, *S. warneri* (Chen et al., 2005) and *A. faecalis* (Shah et al., 2012) have also been recorded as potential textile dye decolorizing bacteria. A recent study of Ekanayake and Manage (2017) recorded 50% decolorization of DB textile dye by *Micrococcus* sp. within 14 days of incubation. However the present study showed complete decolorization of DB dye by the bacterium *M. luteus* indicating that decolorization of textile dyes are species specific. Nevertheless, decolorization of DB was not attributed for the bacteria; *A. faecalis* and *S. warneri* which is studied in the present study.

Table 2. Decolorization of DB dye by selected bacterial strains

Isolate	Decolorization percentage	Time (h)
A. faecalis	CD	60
M. luteus	CD	64
S. warneri	CD	72

*CD: Complete decolorization

Real textile wastewater effluents are a mixture of different colors with a high concentration of COD, BOD, turbidity, and Electric conductivity with high temperature. Bacteria which live in such conditions should be able to tolerate the harsh conditions and must be able to decolorize the dyes effectively without exhaustion. Thus, the decolorization of DB textile dye by the selected bacterial strains was further evaluated under different selected conditions. According to the Sri Lankan wastewater guidelines, the temperature in the effluent water should be less than 40 ^{o}C (BOI, 2011). Kalyani et al. (2008) showed that decolorization of Scarlet R textile dye by *M. glutamicus* is dependent on the variation of temperature applied. Therefore, the effect of temperature on decolorization of DB dye at static condition was evaluated. It was found that over 90% of decolorization of DB dye when temperature changed from 24 to 40 ^{o}C for all three bacterial strains (Figure 1), suggesting the applicability of the selected bacterial strains for practical applications.

The tolerant limit of the pH in textile wastewater effluent should be within the range of 6.0-8.5 at ambient temperature (BOI, 2011). Therefore, decolorization of DB textile dye by selected bacterial strains were evaluated the pH range of 6.0 to 9.0 at 28 ^{o}C under static conditions. Saratale et al. (2009) recorded that the decolorization of most textile dyes by bacterial strains is more effective at pH 7. In the present study, the range of pH 7 to 9 was appeared to be more favorable for all bacterial strains and rapid decolorization of DB textile dye was detected (Table 3). It was found that *A. faecalis* s howed the highest decolorization potential even at slightly alkaline (pH 9) conditions rather than the other two bacteria species. Saratale et al. (2009) recorded the decolorization of an azo dye (Scarlet R) was not possible for both pure and mixed bacterial consortium developed with *Micrococcus* sp. at alkaline pH range of 9 to 12, where above 90% of decolorization was recorded in the present study by the bacterium *M. luteus* contrasting to the previous studies.

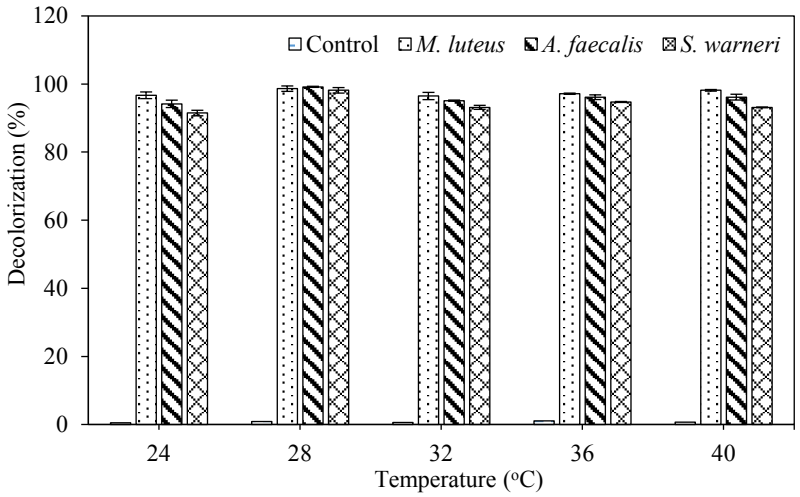

Figure 1. Effect of temperature on decolorization of DB dye by the bacteria: *A. faecalis, M. luteus,* and *S. warneri.* When error bars are not shown, the standard deviation was less than the width of the symbol. (Dotted: *M. luteus*, Diagonal: *A. faecalis,* Diamond: *S. warneri,* Dashed line: Control).

In terms of the effect of dye concentration, complete decolorization of DB textile dye was recorded up to 50 mg/L by all three bacterial strains and descending decolorization trend was found with the increase of the dye concentrations (Table 3). More or less similar result were recorded by other studies suggesting that declining potential of decolorization may be due to the toxic effect of the dye coupled with inadequate production of bacterial biomass (Jadhav et al., 2008; Saratale et al., 2009).

Most textile dyes are deficient in easily available carbon or nitrogen sources in their structures (Padmavathy et al., 2003; Senan and Abraham, 2004). Therefore, decolorization performances by *A. faecalis, M. luteus* and *S. warneri* were evaluated with the supplement of additional carbon or nitrogen sources into the synthetic MSM. In the present study, complete decolorization of DB was obtained using the bacteria: *A. faecalis* and *M. luteus* with the presence of tryptone and peptone in the medium within 48 h of incubation (Table 4). However, supplement of starch, glucose and urea did not play noteworthy role in decolorization of DB dye by the bacteria: *A. faecalis, M. luteus* or *S. warneri*. Saratale et al. (2010) recorded that decolorization of Green HE4BD dye with mixed bacterial consortium consists of *Proteus vulgaris* and *M. glutamicus* were efficient when the synthetic medium was supplemented with tryptone or urea and decolorization was not evident without supplement of any carbon or nitrogen source. Thus, it has been suggested that the efficiency of decolorization depends on carbon or nitrogen sources employed. This may be due to the stimulatory or inhibitory effects on the enzyme of the bacteria that are involved in the degradation of the structure of the textile dyes (Jadhav et al., 2010).

The static condition was more favorable for effective decolorization of DB textile dye as the three strains of bacteria employed in the present study yield complete decolorization of the dye within 72 h while shaking conditions taking more than 14 days for the same conditions (Figure 2). More or similar comparable decolorization patterns were recorded by other studies for decolorization of different textile dyes using bacteria (Moosvi et al., 2007; Shah et al., 2012). Moosvi et al. (2007) recorded that the most of textile dye decolorizing bacterial enzymes such as azo reductase are normally inhibited with the presence of oxygen due to the completion in oxidation of reduced electron carriers with either oxygen or azo groups, supporting the significant relationship between dissolve oxygen and decolorization of DB by *A. faecalis*, *M. luteus*, and *S. warneri* in the present study.

The effect of consecutive addition of DB dye showed that decolorization of DB textile dye was successive up to three consecutive cycles for *A. faecalis*, *M. luteus*, and *S. warneri* and then a descending pattern of decolorization of the dye was observed (Table 5). The declining pattern of decolorization might be due to the reduction of bacterial cells with exhaustion of nutrients. However, results of the present study emphasize the effectiveness of isolated bacterial strains: *A. faecalis, M. luteus,* and *S. warneri* for its practical approaches of bioremediation of textile dyes.

Table 3. Effect of different pH and initial dye concentrations on decolorization of DB textile dye by the bacteria; *A. faecalis, M. luteus,* and *S. warneri.*

Bacterial strain	Decolorization (%) (at 72 h of incubation)							
	pH				DB dye concentration (mg/ L)			
	6	7	8	9	25	50	75	100
Control	0	0	0	0	1	0	0	0
A. faecalis	78	CD	98	93	CD	CD	75	66
M. luteus	60	CD	97	90	CD	CD	81	73
S. warneri	60	CD	96	90	CD	CD	71	58

*CD: Complete decolorization

Table 4. Decolorization of DB textile dye by *A. faecalis, M. luteus,* and *S. warneri* with the presence of different carbon and nitrogen sources.

Bacterial strain	Decolorization (%) (at 48 h of incubation)						
	Without C or N source	Starch	Glucose	Yeast	Tryptone	Peptone	Urea
Control	0	0	0	0	1	1	0
A. faecalis	56	27	57	85	CD	CD	58
M. luteus	64	22	73	91	CD	CD	43
S. warneri	38	21	33	83	95	94	54

*CD: Complete decolorization

Table 5. Repeated addition of textile dye along with different bacteria strains; *A. faecalis, M. luteus,* and *S. warneri*

Bacterial strain	Decolorization percentages at the end of each cycle			
	1st cycle	2nd cycle	3rd cycle	4th cycle
A. faecalis	CD (60 h)	CD (48 h)	98% (48 h)	86% (52 h)
M. luteus	CD (64 h)	CD (48 h)	96% (48 h)	72% (52 h)
S. warneri	CD (72 h)	CD (54 h)	92% (52 h)	68% (58 h)

*CD: Complete decolorization

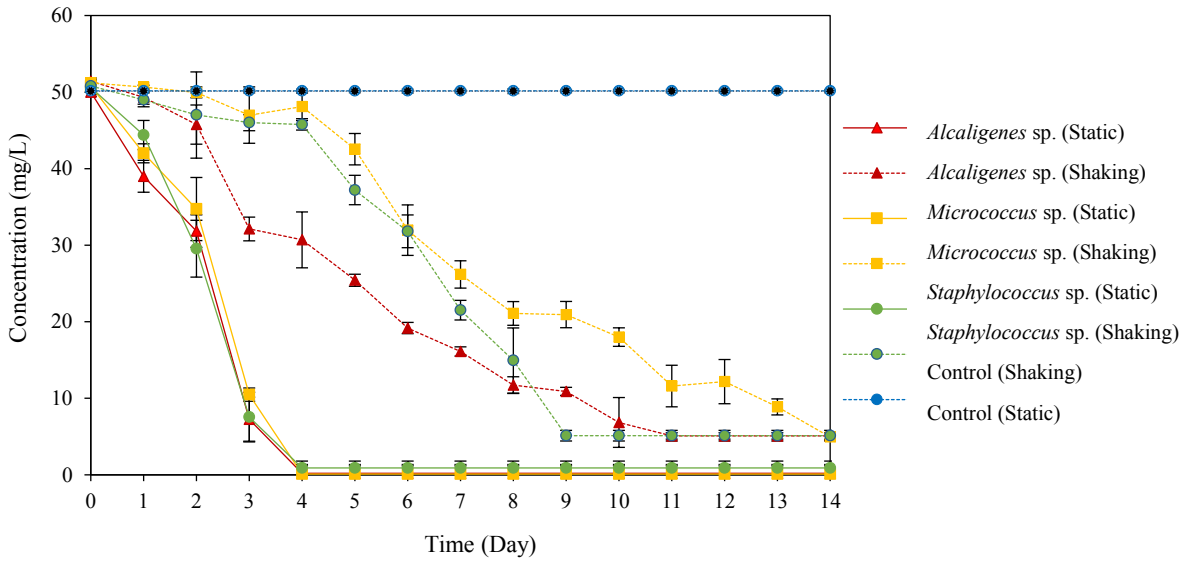

Figure 2. Decolorization of DB textile dye by *A. faecalis, M. luteus,* and *S. warneri* (Closed triangle: *A. faecalis,* Closed squre: *M. luteus,* Closed diamand: *S. warneri,* Closed circle: Control, Solid lines: decolorization at static conditions, Dotted lines: Decolorization at shaking conditions at 100 rpm).

3.3 Phytotoxicity study

Though the untreated textile dye effluent water cannot be used for further uses due to the toxic nature, the treated textile wastewater effluents can be used for agrarian purposes if treatment is possible to remove the toxic nature. In some instances, the secondary byproducts are more toxic in some synthetic chemicals than their original forms (Guruge et al., 2007). Therefore, a seed germination assay was employed to assess the toxicity of the by-products which were generated after the bacterial treatment. In the present study, *O. sativa* and *V. radiate* seeds which were treated with DB dye, showed 8% and 12% germination respectively (Table 6). The resulted low germination percentages of these seeds emphasis the toxic effect which lead to environmental problems when the dyes are released to the environment without any treatment. Interestingly, the seeds treated with the decolorized dye solutions by *A. faecalis, M. luteus,* and *S. warneri*, showed around 100% germination for both *O. sativa* and *V. radiate* indicating the detoxification of DB textile dye by the bacterial treatments.

Table 6. Phytotoxicity of DB textile dye and the bacteria treated water

Bacterial strain	Germination percentage (%)	
	Oryza sativa	*Vigna radiate*
Control (MSM only)	100	100
DB (50 mg/L)	08	12
Decolorized dye by *A. faecalis*	100	100
Decolorized dye by *M. luteus*	100	100
Decolorized dye by *S. warneri*	99	100

4. CONCLUSION

Three potential bacterial strains for decolorization of CI Direct Blue 201 textile dye were isolated from textile wastewater effluent sites in Sri Lanka and identified as *A. faecalis* (MK166784), *M. luteus* (MK166783), and *S. warneri* (MK256311) through their molecular characteristics. *A. faecalis, M. luteus,* and *S. warneri* showed complete decolorization of the DB dye within 60, 64, and 72 h of incubation under static conditions, respectively. The effective temperature range was 24 to 40 ºC where pH ranged from 7 to 9 with the presence of tryptone, peptone or yeast in the medium. Further, phytotoxicity assay confirmed detoxification of the DB textile dye by the bacterial treatment giving 100% seed germination after application of the bacterial treated textile dye effluent water. Therefore, the isolated bacterial strains in the present study can be used as the potential biological treatment agents for future applications in textile dye treatment processes.

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