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Efficient dye decolorization by *Virgibacillus marismortui* B6 and its impact on seed germination

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ABSTRACT

Globally water pollution is a menace and treatment of industrial discharge is a mammoth task. Microbial sources have long been employed for bioremediation of the majority of such hazardous and toxic waste and are probably the only answer to serious environmental issues in years to come. To assess textile dye decolorization 33 bacterial isolates were screened against 4 different textile dyes and effluents. Isolates that showed remarkable ability to decolorize Reactive black B5 dyewere studied for morphological features, biochemical characters, and molecular identification by 16SrDNA sequencing. A critical parameter of assessing the toxic effects on seed germination was studied using wheat (*Triticum aestivum*) and green gram (*Phaseolus mungo*). The present study is aimed at developing a cost-effective and eco-friendly method of textile waste treatment. Affirmative effects of bacteria treated effluents on seed germination indicate futuristic bioremediation processes to be developed and its further implications in agriculture.

Key words: Dye decolorizing bacteria, *Virgibacillusmarismortui,* azo dyes, seed germination, phytotoxicity

Introduction

Synthetic dyes give fabulous results of shades and finishing this makes its use un-avoidable in the era as compared to natural dye. Textile industries are the major ones to use dyes and even have a great part to expel dye polluted water to the environment. This directly affects the consumption of usable water, issues like water crisis and pollution to resources, as the textile industry mainly uses azo dyes which are very toxic to the life forms (Mahmood et al., 2015; Moawad et al., 2003). The serious problem with azo dyes is their degradability as they are recalcitrant, and if degradable itis partialdegradation or secreting more toxic end products.(Lellis et al., 2019). The water thrown out of dyeing industries need to get pre-treatments to reduce the toxicity levels of end products and decrease the percentage of non-biodegradable components of dye from water disposed or reused in agriculture (David Noel and Rajan 2015; Bardi and Marzona 2013).Physical and chemical processes are available wastewater treatments, but they are cost expensive, low degradation levels, and high toxic by-product production.(Shakeel et al., 2020). The alternative way of wastewater treatment before draining in main water resources is biodegradation using microorganisms, which has shown a cost-effective and eco-friendly approach

to rectify the problem(Parmar and Shukla 2015; Saratale et al., 2011). The only disadvantage is the speed or rate of reactions as the dye-containing wastewaterdiscarding values are rapidly increasing and the speed of biodegradation of dye is very slow.(Shukat et al., 2019)

The improvisation of biodegradation speed is bythe addition of specific potential microbial cultures to the wastewater plant to overcome the basic problem of slow bio-degradation process rate. The indigenous species show more potency for the biodegradation work rather than the potent species of other areas (Nasrin et al., 2019; Neetha et al., 2018). To achieve this, there isa needto isolate the potential microbial species from the region of industrial wastewater of dyeing or printing companies. As indigenous species show more potency for biological degradation. Henceit is required to isolatethe microbial species from the area of disposingfor the pre-treatment of dye-containing wastewater (Khan and Malik 2018).

Materials and Methods

Description of the collected sample

Textile dyesReactive black B55 (RbB5, di-azo), Reactive red 120(RR120, di-azo), Reactive Orange 16 (RO16, mono-azo), and Reactive Yellow 145(RY145, mono-azo) were procured from one of the cotton saree printing industry with common effluent treatment plant (CETP) of Jetpur, Gujarat,India with 21.7547° N latitude and 70.6180° E longitude coordinates, wastewater sample was collected with random sampling technique, transported to the laboratory and preserved at 4° C until further process.

Isolation and Screening of dye decolorizing bacteria from the wastewater effluent

A 10%(v/v) industrial effluent was inoculated in 200ml of Nutrient broth containing 500ml Erlenmeyer flask, incubated at room temperature for 48h.10%(v/v) of this preincubated nutrient broth was inoculated in 200ml fresh, Sterile Bushnell and Haas medium(BHM) containing glucose (0.1% w/v) and peptone (0.1% w/v) with 250 ppm of all four dyes mentioned in the description of collected sample in 500ml flask and was incubated at room temperature for 72h. Using serial dilution technique, the enriched BHM media broth was used as samples were spread onto BHM agar plates with varied dye concentrations of 100- 500ppm of all four dyes followed by incubation at 37°±2°C temperature for 24-48 h. Morphologically different isolates were selected and named as B1-B6, R1-R8, Y1-Y12, G1-G7 and were preserved on nutrient agar slants at 4°±2°C.

Screening of Dye decolorizing bacteria

A) Primary screening

Inquest to isolate apotent bacterial isolatewith the ability to decolorize and degrade azo dye, 33 morphologically different bacterial species were primarily screened against each dye with 500ppm concentration. 50 ml BHM with (1% w/v) glucose and peptone was inoculated with the 1%(v/v) of the activated culture in a 150 ml flask. Experimental sets were incubated at room temperature at static conditions. Samples were collected at 0h, 6h, 12h, and 24h respectively.The inoculated broth was thencentrifuged, and supernatants were examined spectroscopically on a respective wavelength of individual dyes usinga UV-Visible spectrophotometer (Shimadzu,Japan). Liquid broth without inoculation was used as a control. Percentage of decolorization was calculatedwith the following formula (Khan and Malik 2018):

%decolorization = [(O.D. of control sample–O.D. of the treated sample)/O.D.of Control]*100

B) Secondary screening

Based onresults of primary screening,4 bacterial isolates viz. R1, B2, B4, and B6 were selected further on their efficiency and rate of decolorization of various textile dyes. The efficiency was assessed asa percentage of decolorization. These four isolates are further studied for their dye decolourizing potential with a set of an experiment consisting of 200ml BHM media containing (0.1 % w/v) glucose, peptone, and all four dyes (RbB5, RR160, RO16, RY145)

with 500ppm concentration in 500ml flasks. Media consisting of all four dyes were inoculated with the primarily screened isolates (R1, B2, B4, and B6) with 5%(v/v) inoculum in each flask and were incubated at room temperature for 96h. An experimental set for consortia analysis with each organism in equal inoculum size (1.25%v/v) was incubated against mixed dye-containing broth. Samples were collected, centrifuged and supernatants were examined using a UV-Visible spectrophotometer at 12h of intervals. Percentage decolorization was calculated for analysis.

All the experiments were performed in triplicate and results were statistically analyzed.The media used were procured from Hi-Media Laboratories, Mumbai,India.

Sugar utilization, Biochemical and Molecular identification of potential isolates

Biochemical characterization is one of the basic techniques for the identification of microorganisms.The selected 33 isolates were examined for various biochemical tests viz. Catalase test, Oxidase test, Methyl Red test, Voges Proskauer test, Citrate Utilisation, Indole Production, Urease Production, Hydrolysis of Casein, Gelatine, Starch, Sugar fermentation: Glucose, Xylose, Maltose, Mannitol, Fructose, Sucrose, Trehalose, Galactose, Lactose, Inositol, Sorbitol, Dextrose, Arabinose, Mannose, Cellobiose, Raffinose, Rhamnose, H_2S Production test, and Nitrate reduction Test.

Molecular identification

The potential 4 isolates were selected and identified by molecular 16S rRNA gene amplification method using 27 F5' (AGAGT TGA TCMTGGCTC A G) 3' and 1492R5' (TACGGYTACCTTGTTACG A CTT) 3'primers for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30µl reaction mixture by using an EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2minutes, 35 cycles of 95°C for 1minutes, 55°C, and 72°C for 1min each were performed, finishing with a 10-min step at 72°C. After successful gene amplification, the PCR products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). A sequencing reaction was performed using a PRISM Big-Dye Terminator v3.1 Cycle sequencing kit. Sequencing primers used were 785F 5'(GGATTAGATACCCTGGTA) 3' and 907R 5'(CCGTCAATTCMTTTRAGTTT) 3'. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5min, followed by 5min on ice, and then analyzedby ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequenced so obtained was phylogeneticallyanalyzed usingBLASTn (http://www.ncbi.nlm.nih.gov/blast) tool (Altschul et al., 1990). The sequence obtained was submitted to the NCBI domain for the required accession numbers.

Seed germination test

The phytotoxic effects were assessed to adjudge the toxicological aspect of dye decolorization if any, by an organism and its further reuse for irrigation. The effect ofReactive black B55 and Reactive Orange 16 dyes and their metabolites on germination and growth of these 2 types of economically important crops were studied. In this study, the effect of two different dyesReactive black B5 and Reactive Orange was evaluated on germination of seeds of 2 crops: Monocot-Wheat (*Triticum aestivum*) and 2) Dicot-Green gram (*Phaseolus mungo*). For the two plant varieties mentioned above, 06 seeds of each were taken with 3 different samples pre-treatment viz.seeds+d/w, seeds+synthetic dye effluent, and seeds+ bacteria treated effluent. These were then placed on Whatman filter paper in a sterilized Petri plate. The regular watering was done by simply wetting the filter paper with 5ml of respective pre-treatment every 12 h continuously for 7 days. All the plates were incubated at room temperature (28±2°C).Six seeds three of each crop plant were soaked and treated with 3 different solutions (distilled water, synthetic dye

effluent, synthetic dye effluent treated with bacterial isolates) were placed on a Whatman filter paper in the sterilized Petri plate. The filter papers were wetted with 5 ml of distilled water (control) or with various concentrations of dye in distilled water every12 hour. All dishes were kept at room temperature (28±2 C) for a period of 7 days. At the end of the germination experiment, the root and shoot length were measured in cm/plant. The results were recorded upto the 9th dayof growth and then compared.

Results and Discussion

Morphological and Microscopic Characteristics of the selected bacterial isolates

Severaldye tolerant bacterial isolates were selected based on different colony characteristics and microscopic examinations summarized in Table 1. Amongst,all 33 bacterial isolates selected, 25 isolates ~~selected,25~~were Gram-positive (04 rod-shaped; 21 cocci), while~~cocci),while~~08 were Gram-negative (06 rod-shaped;02 cocci).Colony characters viz. colony size shape, margin, elevation, texture, opacity, and pigmentation were recorded for all these isolates. It was observed that 08 of the isolates were showing irregular colony shape,the remaining 25 had round to oval-shaped colonies on nutrient agar plates. Moreover, 07~~Moreover,07~~of the isolates had raised colonies,while 05 others have slightly raised colonies and remaining 11 had flat colonies appearance.When the margin was studying it was noted that 10 of the isolates had an uneven margin while the remaining 31 were with an even margin.

The pigmentation of the isolated colonies was studied. A total of 09 isolates showed slightly yellow to brown colored pigmented colonies, while the remaining 24 were either white or off-white. Only 02 isolates had a mucoid appearance while the other 31 showed smooth texture. Lastly, it was observed that 22 isolates showed opaque while 11 had translucent colonies. All the isolates were preserved in Nutrient agar slants and 40%(v/v) glycerol vials under refrigeration for further screening of their dye decolorizing potential.

Primary and Secondary Screening of dye decolourizing bacterial isolates

The efficient dye decolorization needs a faster dye decolorization rate, to select such isolate screening was performed first with four textile dyes against each bacterial isolate. Bacterial isolates B6, B4, B2, and R1 show a high percentage of decolorization than others. All four isolates can decolorize all four treated dyes of 500ppm concentration in 24 hrs. Dye RbB5 is decolorized at 96.3% by B6 isolate with the highest efficiency shown, followed by R1 at 94.5 %, B2 at 74.0%, and B4 at 42.1%. Dye RY145 is decolorized at 89.0% by B2, 83.4% by R1, 80.8% by B6, and 44.2% by B4. Dye RO16 shown an 85.9% to 82.6% range of decolorization by these four isolates. Dye RR120 is decolorized at 84.4% by B2 with highest % decolorization followed by B6 with 82.7%, R1 with 56.9%, and B4 with 53.3 % decolorization (data not shown). Other isolates also showdecolorization of these dyes but with relatively slow rates. The spectral analysis studies of treated dyes with 33 isolates scrutinized four isolates B6, B4, B2, and R1 with potency to decolorize azo-dyes RbB5, RY145, RO16, and RR120 at 500ppm in 24hrs.

Textile industrial effluents are majorly having high concentrations of dyes, sometimes it reaches 1200ppm. Even effluent contains multiple dyes at times, to screen the multiple dye decolorizing property, secondary screening was performed using mixed dye (500ppm each) with four selected potential dye decolorizing isolates and their consortium, observed for 96hrs. At the end of 48 hrs, the consortia gave the highest percentage of decolorization with 61.61% followed by B6 with 50%, B2 34.82, R1 26.79%, and B4 with least 24.11% of decolorization. After 96hrs the spectral analysis shows the best result amongst the above five experimental sets with isolate B6 giving 89.29% decolorization, followed by consortia 71.96%, B2 60.71%, R1 51.79%, and B2 50%. The screening results of mixed dye decolorization

shows isolate B6 with the efficient decolorization ability (Figure 1).

Sugar utilization of dye decolourizing bacterial isolates

The ability of all the bacterial isolates to ferment various sugars was assessed. It was observed that they were able to utilize and ferment sugars in the order mentioned below Glucose = Dextrose = Mannitol = Sucrose = Trehalose = Galactose = Inositol = Sorbitol = Mannose = Cellobiose > Maltose > Xylose = Lactose = Rhamnose > Raffinose > Fructose = Arabinose. For two sugars, Fructose and Arabinose it was a general observation that all the isolated bacteria showed the least preference for these two sugars (data not shown).

Table 1: Cell and colony morphology of dye decolorizing bacteria

Sr. No.	Isolate	Gram Reaction	Cell Shape	Colony Size	Colony Shape	Margin	Elevation	Texture	Opacity	Pigment
1	B1	+ve	Rod	Medium	oval	even	flat	smooth	opaque	white
2	B2	-ve	Rod	medium	Irregular	Uneven	raised	smooth	Trans-lucent	pale yellow
3	B3	-ve	Rod	small	round	even	flat	smooth	opaque	yellow
4	B4	-ve	Rod	medium	round	Uneven	raised	mucoid	opaque	white
5	B5	-ve	Cocci	Small	round	even	raised	smooth	Trans-lucent	brownish-yellow
6	B6	+ve	Rod	medium	Irregular	uneven	flat	mucoid	transluce nt	creamy white
7	R1	-ve	Rod	medium	Irregular	uneven	raised	smooth	transluce nt	pale yellow
8	R2	+ve	Rod	Medium	round	uneven	flat	smooth	opaque	white
9	R3	+ve	Rod	Medium	round	even	flat	smooth	opaque	white
10	R4	+ve	Rod	large	oval	even	flat	mucoid	opaque	off white
11	R5	+ve	Rod	Medium	oval	even	raised	smooth	opaque	white
12	R6	-ve	Rod	Medium	oval	even	flat	smooth	transluce nt	white
13	R7	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
14	R8	+ve	Cocci	Small	round	even	raised	smooth	transluce nt	pale yellow
15	Y1	+ve	Cocci	Small	round	even	raised	smooth	transluce nt	brownish-yellow
16	Y2	+ve	Rod	Medium	Irregular	uneven	flat	smooth	opaque	off white
17	Y3	-ve	Cocci	Small	round	even	raised	smooth	transluce nt	yellow
18	Y4	+ve	Rod	Medium	Irregular	uneven	flat	smooth	opaque	off white
19	Y5	+ve	Cocci	Small	round	even	raised	smooth	transluce nt	pale yellow
20	Y6	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
21	Y7	+ve	Rod	Medium	oval	even	flat	smooth	opaque	white
22	Y8	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
23	Y9	+ve	Cocci	Small	round	even	raised	smooth	transluce nt	brownish-yellow
24	Y10	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
25	Y11	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white

25	Y11	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white
26	Y12	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
27	G1	+ve	Rod	Medium	oval	even	flat	buttery	opaque	dirty white
28	G2	-ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
29	G3	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white
30	G4	-ve	Cocci	Small	round	even	raised	smooth	transluce nt	brownish-yellow
31	G5	+ve	Rod	Medium	round	even	flat	smooth	opaque	dirty white
32	G6	+ve	Rod	Medium	oval	even	raised	smooth	opaque	white
33	G7	+ve	Rod	Medium	irregular	uneven	flat	smooth	opaque	off white

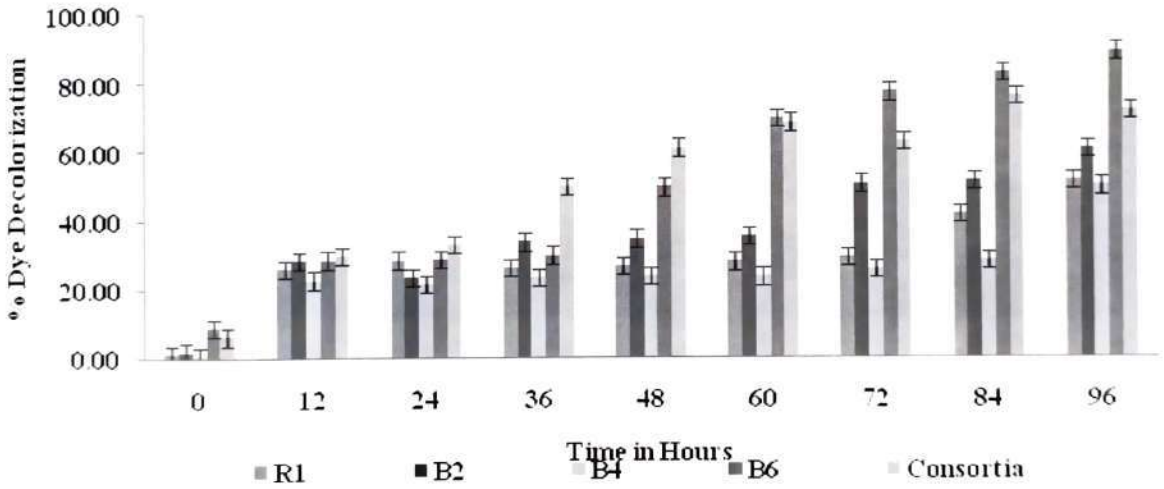

Figure 1: Secondary screening results of selected 4 bacterial isolates and their consortia against mixed dye

Biochemical and Molecular Characterization of the dye decolorizing bacteria

Biochemical characterization of all 33 isolates was studied. The majority of selected cultures showed positive results for the catalase and oxidase test. 06 cultures were showed positive results for citrate utilization,while all others were either negative or showed no detectable results. All of the isolates were positive for Indole production. All isolates enriched and obtained from yellow dye were showing positive results for urease production while all other isolates were urease negative. For nitrate utilization,21 cultures showed positive results while the remaining 12 were negative. When utilization of complex sources of carbon was assessed it was observed that as high as 26 bacterial isolates were able to utilize starch, 17 were able to hydrolyzecasein and only 11 isolates were able to utilize gelatine. For H_2S production also it was observed that only 11 isolates were able to do so. A detailed result is summarized in Table 2.

Four bacterial isolates that were specifically selected from a secondary screening of dye

decolorization and had enormous potential to degrade various dyes selected in the present study were identified by 16S rDNA gene homology analysis. As mentioned in materials and methods, the ~1500bp 16S rDNA gene was amplified by using universal primers. The sequence data were further analyzed for finding the closest homologs for the microbe by comparing gene sequences with reference strains. The nucleotide sequence for all four isolates has been submitted to the GenBank database with accession numbers MF772441-MF772444. Based on nucleotides homology and phylogenetic analysis the microbes were identified. Two of the isolates B2 and R1 were identified and had the closest homology with *Enterobacter cloacae*, isolate B4 as *Leclerciaadecarboxylata* and isolate B6 as *Virgibacillusmarismortui*.Phylogenetic trees were constructed for all isolates using Neighbour Joining Method with 0.75 maximum sequence difference using an online tool from NCBI nucleotide BLAST tool(Figure 2).

Seed Germination test

In this study, it was found that the seeds germination of wheat, as well as mung,was significantly affected by the textile dyes and effluent (Nasrin et al., 2019; Moawad et al., 2003). However, post-treatment of the effluent with potential dye decolorizing bacterial isolate, the

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seeds which indicates that such bacterial remediation approach can be useful in the recycling effluents and other industrial wastewaters (Nasrin et al., 2019). Additionally, it is a good approach for a sustainable environment and for the betterment of irrigation in developing countries where the majority of agriculture is dependent on rain (Vora et al., 2007). For such areas where water scarcity is substantial and alternative sources of water for irrigation are sought, there such a bioremediation approach mediated by bacterial treatment would render the effluents detoxified and nullify the toxic effects of textile dyes (Khan and Malik 2018).

Table 2 Biochemical characterization of selected dye decolorizing bacteria

Sr. No.	Isolate	Catalase	Oxidase	Methyl Red	Voges Proskauer	Citrate Utilisation	Indole Production	Urease Production	Casein	Gelatine	Starch	H_2S Production	Nitrate reduction
1	B1	+	+	+	-	-	+	-	-	-	+	+	+
2	B2	+	+	+	-	-	+	-	+	-	+	-	+
3	B3	+	-	-	-	+	+	-	-	-	+	-	+
4	B4	+	+	+	-	+	+	-	+	+	+	+	+
5	B5	-	+	-	-	-	+	-	+	-	+	-	+
6	B6	+	+	-	-	-	+	-	+	+	+	+	+
7	R1	+	+	+	-	-	+	-	-	-	+	+	+
8	R2	+	+	+	-	+	+	-	+	-	+	+	+
9	R3	+	-	+	+	-	+	-	-	-	-	+	+
10	R4	+	+	+	-	-	+	-	-	-	-	+	-
11	R5	+	-	+	-	-	+	-	-	+	+	+	-
12	R6	-	+	-	-	-	+	-	+	+	+	+	-
13	R7	+	+	-	-	-	+	-	-	-	+	+	+
14	R8	+	+	-	-	-	+	-	-	-	-	+	+
15	Y1	+	+	-	+	-	+	+	-	-	+	-	+
16	Y2	+	+	-	+	-	+	+	+	+	+	-	-
17	Y3	+	+	+	+	-	+	+	-	+	+	-	-
18	Y4	+	+	-	+	-	+	+	+	+	-	-	+
19	Y5	+	+	+	+	-	+	+	-	+	+	-	-
20	Y6	+	+	+	-	-	+	+	+	-	+	-	-
21	Y7	+	+	+	-	-	+	+	+	-	+	-	-
22	Y8	+	+	-	-	-	+	+	+	-	+	-	+
23	Y9	+	+	+	-	-	+	+	-	-	+	-	+
24	Y10	+	+	+	+	-	+	+	+	-	-	-	+
25	Y11	+	+	+	-	+	+	+	+	+	+	-	-
26	Y12	+	+	+	-	-	+	+	+	+	-	-	-
27	G1	-	-	-	-	-	+	-	+	-	+	-	+
28	G2	+	+	+	-	nd	+	-	+	-	+	-	+
29	G3	+	-	nd	-	nd	+	-	-	-	+	-	+
30	G4	+	+	+	-	nd	+	-	-	-	-	-	+
31	G5	-	+	nd	-	+	+	-	-	-	+	-	+
32	G6	-	-	nd	-	nd	+	-	+	+	+	-	-
33	G7	-	-	nd	-	nd	+	-	-	-	+	-	-

+: A positive result -: A negative result nd: results not detected

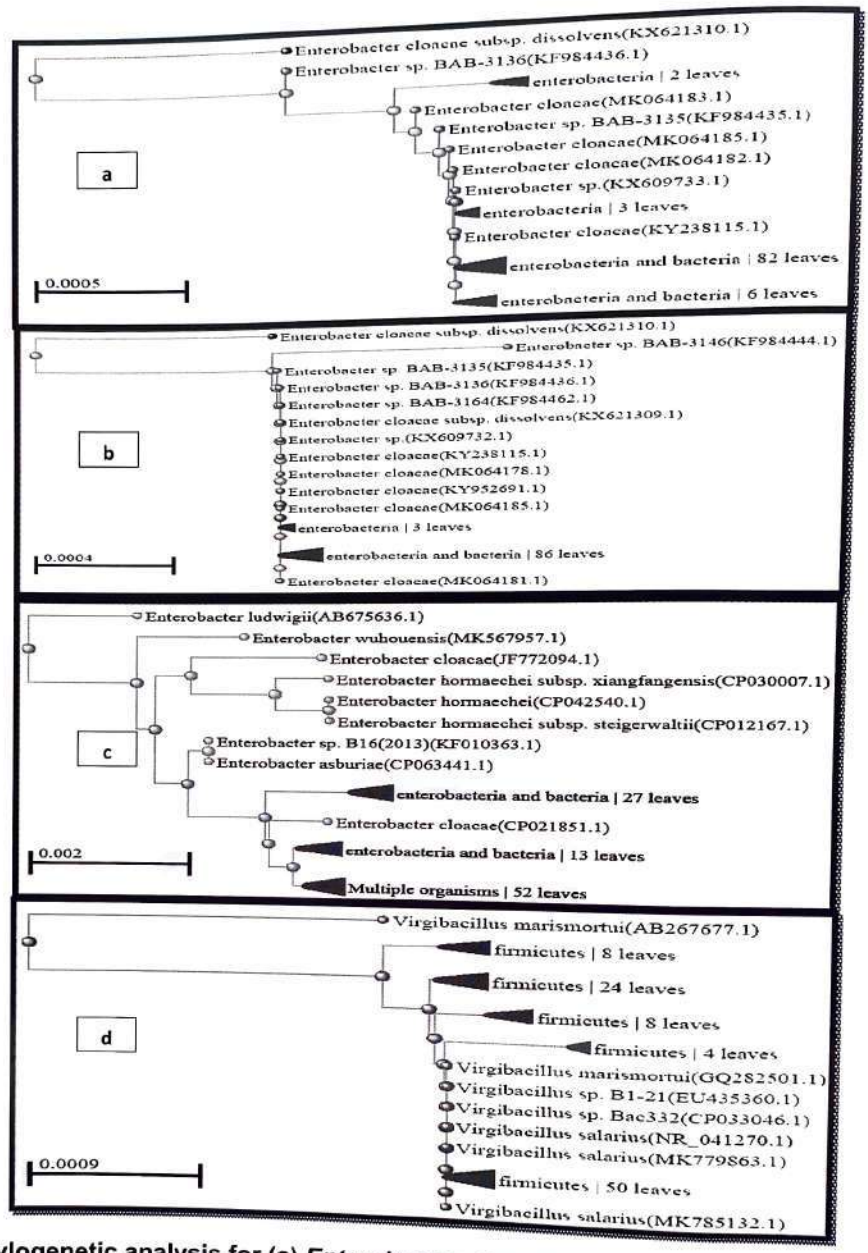

Figure 2: Phylogenetic analysis for (a) *Enterobacter cloacae* B2 (b) *Enterobacter cloacae* R1 (c)*Leclerciaadecarboxylata*B4 (d)*Virgibacillusmarismortui*B6

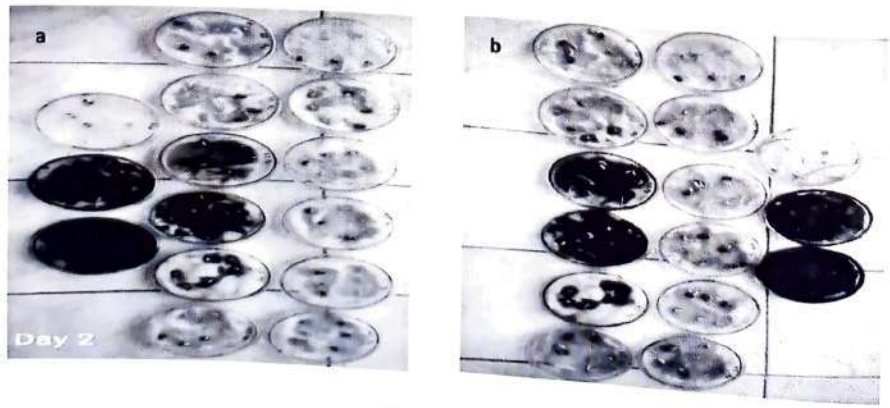

Figure 3: Effect of dyes (effluent) and bacteria treated effluent on germination of seeds 2nd day (a) L-R Lane 1 Control with distilled water and dyes, Lane 2 Seeds treated withReactive black B5, Lane 3 Seeds treated with Reactive Orange, and 9th day (b)L-R Lane 1 Seeds treated withReactive black B5, Lane 2 Seeds treated with Reactive Orange, Lane 3 Control with distilled water and dyes.

Conclusion:

The study on azo dye decolorization and seed germination test for treated dye sample conclude that it's promising for recycling of azo dye polluted water to detoxify it with bacterial isolate B6 identified as *Virgibacillusmarismortui,* and can be used further for irrigation with supportive studies. This shows more than 90% of decolorization at 500ppm of dye concentrations which makes it useful for industrial waste treatments as their dye concentrations expelled are in such range. The non-phytotoxic nature of the treated dye sample makes it moreencouragingto be used for bioremediation.

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