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A methodology - for Isolation of dye degrading bacterial strains

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Abstract: **synthetic dyes pollution spreading everywhere, isolation and identification of dye degrading bacterial strains is a difficult process, it need several methods, screening, identification by biochemical and molecular methods, In this direction we isolated three strains of bacteria which were capable of maximum decolorisation and degradation of Reactive red-11 a synthetic dye, derived from the naphthalene as raw material, contains more than one sulphonic acid groups confirms the recalcitrant and xenobiotic character of dye. Work is proceeded with isolating novel dye degrading bacterial strains by various samples, through that efficient bacterial strains were isolated namely- *Enterococcus casseliflavus* CMGS-1, *Lysinibacillus boronitolerans* CMGS-2, *Klebsiella oxytoca* CMGS-3, were selected dye efficient when results were analyzed and through 16srRNA sequencing analysis method and sequence deposited in GenBank with an accession number-, KT602856, KT602857, KT602858 and also organism deposited in the IMTECH (Chandigarh) with an MTCC num-12538, 12531 and 12532 respectively.**

Keywords: **dye degrading bacterial strains, biochemical, molecular methods.**

1. INTRODUCTION

Biological treatment, either aerobic or anaerobic, is generally considered to be the most effective means of removing the bulk of pollutants dye effluents from water. Also, microorganisms are known to play a crucial role in the mineralization of biopolymers and xenobiotic compounds. Various reports were reported variety of synthetic dyes completely treated, found using microbes, under aerophilic, or microaerophilic and anaerobic systems (Waghmode *et al.*, 2012).

Bacterial strains performs fastest decolrisation and degradation (Tapia-Tussell *et al.*, 2011). These strains having capacity to mineralize dye in single or in mixture of cultures (Cherriaa *et al.*, 2012). Bacterial strains were widely using in the decolorization process due to its replication cycle and gene arrangements, which help in the dye decolozisation completely (Vigneswaram *et al.*, 2010). So for many bacterial strains isolated from soils, contaminated soils and industrial effluents were employed for the degradation/decolorisation of various synthetic dyes individually and in mixture. Till date very few such isolates were used commercially to treat the color effluents viz., *Enterococcus faecalis* YZ66 used for treating Reactive Orange II (Sahasrabudhe *et al.*, 2014). *Enterobacter agglomerans*, Methyl Red (Keharia and Madamwar, 2003). Biodegradation of reactive textile dye Red BLI by an isolated bacterium *Pseudomonas* sp. SUK1 (Kalyani *et al.*, 2008). *Bacillus subtilis* for Acid blue 113 (Gurulakshmi *et al.*, 2008). *Brevibacillus laterosporus* MTCC2298 for Navy blue 3G (Jirasripongpun *et al.*, 2007). *Bacillus fusiformis* KMK-5 for Acid Orange 10 and Disperse Blue 79 (Kolekar *et al.*, 2008). Treatment of synthetic dye became a global concern issue which is creating major factor for pollution.

2. MATERIALS AND METHODS

Dyes and Chemicals

Selection of dyes from various industries were procured , A total of six Reactive Azo dyes were procured from Sigma Aldrich (U.S.A), Heena and Colorise textile industries (Gujarat, India). They are Reactive Blue-4, Reactive Yellow-86, Reactive Navy Blue-59, Reactive Orange-16, Reactive Violet-1, and Reactive Red-11. All these dye were industrial grade and widely used in the textile industries. Out of these Reactive Red-11 (RR-11) widely used azo dye, was selected for the dye degradation studies. All required chemicals, solvents and reagents used in the study were of analytical grade and were procured from, reputed firms (Hi-Media Laboratories Pvt. Ltd., Mumbai; S.D. Fine Chemical Ltd., Mumbai).

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Collection of Samples

As showed in Table-1, soil and effluent samples were collected from the textile industrial area and textile effluent treatment unit, MIDC, Solapur, Maharashtra. Samples including untreated textile mill effluent, sludge soil, dye contaminated soil, greasy soil, colored charcoals from the textile industry, untreated synthetic dye contaminated water. Further some samples were collected from in and around of Kalaburagi, like dye contaminated soil from the M.S.K. Mill area, sewage samples, garbage soil, garden soil. Soil samples from Sharanabasaveshwara lake, Ganesh statue making place (colored soil), market area, dairy unit were also collected. All the samples were collected in a clean sterile bottle and other containers and brought to the laboratory. All the collected samples were processed immediately in the labs if delayed stored in the refrigerator (4^0C).

Table 1: Details about sources of sample, sample inoculums, incubation time and number of isolates from the decolorizing medium containing 100 mg/L RR-11 and 20 mL of processed sample

Sample No.	Kind of samples and their sources	Morphology of sample	Time For Change In Visible Color	Number of bacteria isolated
1	Water containing colour effluents, MIDC area, Solapur.	Colored water sample	12 days	1
2	Soil-surroundings of Ghanate textile industry, Solapur.	Black soil	15 days	2
3	Sludge soil- MIDC area, Solapur.	Wet soil	21 days	3
4	Soil sample of MIDC area, Solapur.	Dry soil	7 days	5
5	Colored wet soil, MIDC area, Solapur.	Wet soil	15 days	3
6	Greasy soil, inside textile industries, Solapur.	Oily soil	No visible Changes	Nil
7	Charcoals-inside textile industry, Solapur.	Black colored solid kind	30 days	1
8	Soil sample, outside textile industrial area, Solapur.	Upper soil	No visible Changes	Nil
9	Soil sample of textile industry, Solapur.	Dye amended soil	No visible Changes	Nil
10	Soil sample of textile industry, Solapur.	Five inches deep soil	No visible Changes	Nil
11	Soil sample of textile industry, Solapur.	Colored soil	20 days	3
12	Wet soil of textile industry, Solapur.	Black colored	No Changes	Nil
13	Dye contaminated soil of textile industry, Solapur.	Colored soil	No visible Changes	Nil
14	Soil sample of near textile dye discharging lake.	Colored soil	30 days	1
15	Sewage water contains dye effluent.	Colored water	No visible Changes	Nil
16	Soil sample of textile industry.	Black color	No visible Changes	Nil
17	Water effluent of textile industry.	Colored water	No visible Changes	Nil
18	Soil sample of textile dye treatment unit.	Bluish colored soil sample	16 days	4
19	Clay soil of textile dye treatment unit.	Black color	No visible Changes	Nil
20	Soil sample of textile dye treatment unit.	Bluish colored	18 days	2
21	Effluent water of textile dye treatment unit, Solapur.	Colored water	14 days	2

22	Soil sample of textile dye treatment unit, Solapur.	Bluish colored sample	5 days	2
23	Dye contaminated soil of M.S.K Mill area Kalaburagi	Colored soil	No visible Changes	Nil
24	Soil sample of M.S.K Mill area Kalaburagi	Black colored	No visible Changes	Nil
25	Soil sample of M.S.K Mill area Kalaburagi	Black colored	No visible Changes	Nil
26	Soil sample of M.S.K Mill area Kalaburagi	Black colored	No visible Changes	Nil
27	Soil sample of M.S.K Mill area Kalaburagi	Black colored	No visible Changes	Nil
28	Sewage water of Naganalli road Kalaburagi	Plain Water	No visible Changes	Nil
29	Sewage water of Naganalli road Kalaburagi.	Plain Water	No visible Changes	Nil
30	Agriculture soil of Naganalli road Kalaburagi .	Black colored	No visible Changes	Nil
31	Sewage water of R.T.O., cross road Kalaburagi.	Sewage Water	No visible Changes	Nil
32	Sewage water of R.T.O., cross road Kalaburagi.	Sewage Water	No visible Changes	Nil
33	Soil sample petrol pump, Near Sedam ring cross, Kalaburagi.	Greasy soil	No visible Changes	Nil
34	Sewage sample, from University hostels.	Sewage sample	No visible Changes	Nil
35	Brown colored soil, Gulbarga University garden.	Brown color	No visible Changes	Nil
36	Soil sample of Microbiology Dept. G.U.K.	Black color	No visible Changes	Nil
37	Soil sample of Sharanabasaveshwar Lake, Kalaburagi .	Black color	No visible Changes	Nil
38	Water sample of Sharanabasaveshwar Lake Kalaburagi.	Plain water	No visible Changes	Nil
39	Garbage Dump yard soil of Kusnoor road Kalaburagi.	Black color	No visible Changes	Nil
40	Soil sample of Vegetable market, Kalaburagi.	Black color	No visible Changes	Nil
41	Soil samples of Vegetable market Kalaburagi.	Black color	No visible Changes	Nil
42	Soil sample of KMF dairy unit area, Kalaburagi.	Black color (discharging unit)	No visible Changes	Nil
43	Water sample of KMF dairy unit area, Kalaburagi	Water (discharging unit)	No visible Changes	Nil
44	Effluents of Cloth dying units area, Kalaburagi.	Colored water	No visible Changes	Nil
45	Soil sample of Cloth dying Units area, Kalaburagi.	Black soil	No visible Changes	Nil
46	Soil sample of Ganesh statue making shop Kalaburagi.	Colored soil	No visible Changes	Nil
47	Water sample from Ganesh statue making place, Kalaburagi.	Colored water	No visible Changes	Nil

3. METHODS

Preparation of Media and Reagents

The mineral salt medium (MSM) was prepared as per (Brilon *et al.* 1981) with some modification. MS medium was prepared by adding 10 mL of solution-2 to 100 mL of solution-1and adjusted pH-7.0. The solution-1 was prepared by adding gms/L of $Na_2HPO_4.2H_2O$ (12.00), KH_2PO_4 (2.00), NH_4NO_3 (0.50), $MgCl_2.6H_2O$ (0.10), $Ca(NO_3)_2.4H_2O$ (50.00 mg), $FeCl_2.4H_2O$ (7.50 mg) to 1000 mL distilled water. The solution-2 (trace element solution) was prepared by adding mg/L of $FeSO_4.7H_2O$ (0.10), $MnCl_2.4H_2O$ (3.0), $ZnSO_4.7H_2O$ (10.0), $CuSO_4.5H_2O$ (1.0), $MnSO_4.H_2O$(0.017), $NiCl_2.6H_2O$ (2.0), $Na_2MoO_4.2H_2O$ (3.0), H_3BO_3 (30.0), $CuCl_2.2H_2O$ (1.0) into 1000 mL of distill water. Further, MSM was blended with different concentrations of RR-11or RO-16 separately and used as decolorizing medium (DM) throughout the study and un-inoculated flasks were also incubated as control. The MS with 1.8% agar was used for isolation and maintenance of pure culture. The media were sterilized at 121^0C for 20 min before use.

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Isolation and screening for dye decolorizing bacterial isolates

For the isolation process the protocol referred which is prescribed in Anjaneya *et al.* (2011). Ten grams of soil sample or 10 mL of water sample (turbid) were added to 100 mL normal saline (0.9%) containing in 500 mL conical flasks containing 100 mL of normal saline and kept on rotary shaker at 120 rpm for one hour and left at room temperature without shaking until all suspended particles were settle down. The supernatant was used for the screening of RR-11 decolorizing microorganisms. Twenty ml of supernatant was inoculated to 100 mL Mineral Salt Medium (MSM) containing 50 mg/L RR-11 as sole source of carbon and incubated at 35^0 C till visible color changed in the flask . The flasks showing more than 50% reduction in the color intensity were selected and decolorisation was confirmed by UV-Vis spectrophotometer taking optical density at 540 nm. Again 20 mL of declorized culture was inoculated into fresh 100 mL DM (Decolorizing medium) containing flasks and were incubated once again and observed for the more than 50% of reduction in the initially added dye. Again the flasks showing maximum decolorization were selected for the isolation of RR-11 decolorizing microorganism. A 0.25 mL of culture from decolorized those flasks was taken out and inoculated on the MS agar medium containing 50 mg/L of RR-11 by pour/spread plate method. The plates were incubated at 35^0 C till visible growth appeared on the plates. The colonies showing clear zones around them were picked up and streak on the nutrient agar plates and study the cultural and morphological characteristics. Further physiological and biochemical tests were performed to identify isolate up to genus level. The characterized cultures were subculture on MS agar containing 0.1% yeast extract and 100 mg/L RR-11 slants and after growth two slants were stored at 4 ^{0}C after adding 25% of sterile glycerol on the culture surface and one slant used for the further study.

Screening for dye decolorizing potential bacterial isolates

Among the bacterial strains isolated from the decolorized flask a total of 30 morphologically different bacterial strains were individually tested their ability of RR-11 decolorisation. The mineral salt medium was blended with RR-11 (50 mg/L) and sterilized at 121^0C for 20 min. The pure bacterial isolates were grown in nutrient broth for 18 hrs, than 10 mL of culture was inoculated into 100 mL of DM and were incubated at 35^0 C without shaking. The decolorization in each flask was routinely checked by measuring the dye concentration in the supernatant of culture at 540 nm for RR-11. The flasks showing maximum decolorization of RR-11 within less incubation time were selected for the further study (Table.2).

Table 2: Isolation of RR-11 decolorizing bacteria from different samples and selection of potent isolated bacterial strains based on their percent RR-11 decolorization.

Sample No.	Bacterial Isolates	Sources	Duration	% Decolo-rization	Selected bacterial strain
Sample-1	Isolate -1	Dye effluent containing water	12 days	45%	No
Sample-2	Isolate -2	Dye effluent containing water	12 days	68%	No
	Isolate- 3	Ghanate textile industry soil	15 days	79%	No
	Isolate- 4	Ghanate textile industry soil	10 days	88%	**CMGS-5**
Sample-3	Isolate -5	Area of MIDC, Solapur.	21 days	45%	No
	Isolate -6	Area of MIDC, Solapur.	13 days	95%	**CMGS-6**
	Isolate -7	Area of MIDC, Solapur.	21 days	64%	No
Sample-4	Isolate-8	Area of MIDC, Solapur.	12 days	60%	No
	Isolate-9	Area of MIDC, Solapur.	6 days	89%	**CMGS-7**
	Isolate-10	Area of MIDC, Solapur.	23 days	56%	No
	Isolate-11	Area of MIDC, Solapur.	26 days	36%	No
sample-5	Isolate-12	Area of MIDC, Solapur.	29 days	13%	No
	Isolate13	Area of MIDC, Solapur.	14 days	67%	No
	Isolate14	Area of MIDC, Solapur.	21 days	76%	No
	Isolate- 15	Area of MIDC, Solapur.	28 days	40%	No
Sample-7	Isolate -16	Inside area of textile industry	23 days	35%	No
Sample-11	Isolate-17	Surrounding of textile industry	3 days	95%	**CMGS-12**
	Isolate-18	Surrounding of textile industry	7 days	78%	**CMGS-4**
	Isolate-19	Surrounding of textile industry	18 days	81%	No
Sample-14	Isolate-20	Lake, near textile effluents	24 days	56%	No

		discharging area.			
Sample-18	Isolate-21	Textile dye treatment unit	6 days	96%	**CMGS-2**
	Isolate-22	Textile dye treatment unit	9 days	89%	No
	Isolate-23	Textile dye treatment unit	6 days	91%	**CMGS-11**
	Isolate-24	Textile dye treatment unit	2 days	96%	**CMGS-3**
Sample-20	Isolate -25	Textile dye treatment unit	8 days	86%	**CMGS-8**
	Isolate -26	Textile dye treatment unit	11 days	91%	**CMGS-9**
Sample-21	Isolate-27	Textile dye treatment unit	22 days	34%	No
	Isolate-28	Textile dye treatment unit	11 days	77%	No
Sample-22	Isolate-29	Textile dye treatment unit	5 days	92%	**CMGS-10**
	Isolate-30	Textile dye treatment unit	3 days	95%	**CMGS-1**

Characterization and identification of dye decolorizing bacterial isolates

The isolated bacterial strains were identified up to the genus level by comparing their cultural, morphological, staining, biochemical and physiological properties with characters mentioned in the Bergey's manual of systematic bacteriology (Holt *et al.*, 1994)

Cultural characters on culture medium

A loop full culture was streaked by quadrant streak method, on nutrient agar plates was incubated at 35^{o} C for 24 hrs. After incubation the pure isolated colonies were selected for the cultural characters like colony size, shape, color, texture and margin. Cell arrangement were performed by Grams staining, Cell morphology, motility and spore staining also were performed.

Biochemical tests

Biochemical tests like Indole, Methyl red, Voges Proskaur, Citrate utilization, gelatin hydrolysis, Urease, Starch utilization, Catalase tests, were performed for the characteristic identification of isolates. The biochemical profiling of isolates was also performed by doing sugar utilization test by using Hi-Carbo kit (Hi-Media Lab. Pvt. Ltd., Mumbai, India).

16s rRNA sequence analysis

Pure bacterial culture of three decolorizing isolates were sent for the 16s rRNA sequencing at Royal Life Sciences Pvt. Ltd., Hyderabad, India, procedure for 16S rRNA sequence analysis.

Procedure-1. The genomic DNA was extracted by Insta Gene Matrix bacterial DNA purification kit (Bio-Rad, USA) and PCR protocol done according to the manufacturer's instructions.

The universal primes for 16s rRNA sequence amplification used were with a forward primer, Eub-27F (5'-3': AGA GTT TGA TCC TGG CTC AG) and a reverse primer, Eub1492R (5'-3': ACG GCT ACC TTG TTA CGA CTT) the action produced were of approximately 1500 bp. The PCR performed by an initial denaturation at 95^{0} C for 10 min, then the DNA was amplified for 25 cycles and with each cycle performed denaturation at 95 ^{0}C for 1min, annealing at 55 ^{0}C for 1 min and extension at 72^{0} C for 1.5 min and final extension was done for10 min. Then PCR products were purified using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). A resultant product approximately of 1500 bases was compared with closely related taxa of the isolate, retrieved from the GenBank database using BLAST (blastn) program on the NCBI website (http://www.ncbi.nlm.nih.gov). The alignment of the sequences was done using CLUSTAL W program V1.6 at European bioinformatics site (http://www.ebi.ac.uk/Tools/msa/). The sequence was refined manually after cross checking with the raw data to remove ambiguities and submitted to GenBank. Evolutionary history checked to see the phylogenetic position of bacterial isolate using the neighbor-joining method (Saitou, 1987). The Phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takazeki *et al.*, 1995).

The clock calibration to convert distance to time was 0.02 (time/node height). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the Phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura and Nei, 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 software as per Tamura and Nei, (2014).

Decolorisation assay

Calculation of absorption spectrum of different Reactive azo dyes

The dye concentration was calculated by using UV-Vis spectrophotometer. A solution of each reactive dye was prepared by dissolving a known amount of dye in 100 mL of distilled water and maximum absorbance was determined by scanning between 200-900 nm in UV-Vis spectrophotometer. Then λ_{max} of each reactive dye used in this study was determined.

Preparation of Pre-Inoculum

A loopful of isolated colonies of testing isolate was picked from the fresh growth on MS agar medium was inoculated into MS liquid containing dye supplemented with yeast extract (0.1%) and incubated under static condition until complete decolorization of dye occurred. This culture was used as pre-inoculum and fresh inoculum was prepared for each set of decolorisation experiments from the mother culture.

Decolorisation experiments

The dyes decolorisation experiments were performed by adding 50 mg/L of RR-11 or RO-16 to plane MSM (without any carbon source) and MS broth supplemented with yeast extract (0.1%). In the preliminary screening for dye degrading bacteria plane MS broth alone for that confirm the active bacteria utilized dye as sole source of carbon for their energy and growth. Co-substrates like yeast extract enhanced the decolorization process by acting as carbon and nitrogen sources (Kadpan *et al.*, 2000; Jain *et al.*, 2012). After the isolation of decolorizing bacteria from natural samples, further decolorisation experiments were performed using 0.1% of yeast extract as co-substrate. The flasks containing 100 mL decolorizing medium with 0.1% yeast extract were inoculated with required inoculum of each bacterial isolate and uninoculated flasks as controls. The flasks were incubated at 35^0 C under static as well as under shaking (120 rpm) conditions till the decolorisation was completed. The 5 mL of cultures were withdrawn at different intervals of incubation period to check the initial dye concentration in the culture medium. The supernatant was collected by centrifugation at 10000 rpm for 15 min and O.D. was measured at 540 nm in case of RR-11 and at 500 nm for RO-16 using UV-Vis spectrophotometer (model Systronics, AU-2700) and compare test OD with control one and calculate the percent decolorisation of initially added dye using the following formula (Dave and Dave, 2009).

Decolorization=initial optical density-final optical density×100/initial optical density

Checking of decolorization is not due to adsorption, adsorption or change in pH

To confirm whether decolorisation was due to microbial action or absorption/adsorption or change in pH, suitable tests were performed. To check whether decolorization is due to adsorption by washing centrifuged cells pallet of decolorized culture by several times with water or methanol or ethanol or acetone and was measured for the presence of dye at their respective λ_{max} using spectrophotometer. Similarly for absorption, cell pallet was sonicated and cell free extract was prepared and presence of dye was checked by spectrophotometric analysis. Whether decolorization is due to change in pH was confirmed by altering the pH of culture filtrate using 0.1N NaOH/HCl.

Conclusions- through the methods , isolated various dye degrading bacterial strains , maximum decolorisation strains further proceeded with optimization influence factors, finally with confirmation with dye degradation by Extraction of metabolites (dye intermediates) during decolorizing process by bacterial isolates through Thin Layer Chromatography (TLC), Fourier transform infrared spectroscopy (FT-IR) analysis, Liquid chromatography – Mass Spectrometry (LC-MS) analysis, which gave a clear identification produced metabolites and dye degradation.

4. CONCLUSIONS

Textile industries are huge producers of effluents, which cannot be easily treated , physical and chemical methods fail in proper detoxifications of these dyes, because synthetic dyes are highly soluble, present article reveals the proper isolation and methodology for the isolation of microorganisms , which are the natural cleaners of nature, this article helps the researchers who working n the field of degradations synthetic yes and other effluents.

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