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A Study on Isolation and Characterization of Dye Degrading Microorganism

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Abstract

Over the past decades, biological decolorization has been investigated as a method to transform, degrade or mineralize azo dyes. Moreover, such decolorization and degradation is an environmentally friendly and cost competitive alternative to chemical decomposition possess. Therefore, present study was aimed for isolation and identification of dye degrading microorganisms. The effluent from textile industry was screened for dye decolorization potential. Further, morphological and biochemical characterization was carried out. Results revealed that three isolates *viz.* IS 1, IS 2, and IS 3 shown maximum decolorization percentage at 150 mg/mL concentration. However, among the three isolates (IS 1, IS 2, and IS 3), IS 2 shown maximum decolorization potential of selected dyes at the concentration of 150 mg/mL, and hence IS 2 was selected for further morphological and biochemical characterization studies. IS 2 was positive for catalase, oxidase, methyl red, Voges Proskauer, nitrate utilization, urease activity, casein hydrolysis, and starch hydrolysis tests. Whereas, the isolated strain (IS 2) was negative for indole, citrate utilization, and CAMP tests. The isolated bacterial strain (IS 2) was grown on *Bacillus cereus* agar plan, and hence IS 2 strain was

identified as *Bacillus sp.* In conclusion, newly isolated *Bacillus sp.* could be used as a good tool for bioremediation of various textile effluent treatment by converting toxic dye into colorless, harmless product., the treated effluents can be reused for textile industry.

Keywords:*Bacillus sp.*Azo dye, Effluent, Decolourization, Textile industry

Introduction

Environmental pollution has been recognized as one of the major hazards of the modern world. Due to the rapid industrialization, lot of chemicals including dyes manufactured and used in day-to-day life.[1] The presence of very small amount of dye in water (<1 ppm) is highly visible, affecting the aesthetic merit, water transparency and gas solubility in lakes, rivers and other water bodies.[2]The effluents from these industries arecomplex, containing a wide variety of dye products such as dispersant, acids, bases, salts, detergents and oxidants. Discharge of these colored effluents into the rivers and lakes reduce dissolved oxygen concentration, thus creating anoxic conditions that are lethal to resident organisms.[3] India's dye industry produces every type of dyes and pigments. Production of dye stuff and pigments in India is close to 80,000 tones. India is the second largest exporter of dye stuffs and intermediates after China. The textile industry accounts for the largest consumption of dyestuffs, at nearly 80%.[4]

The discharge of dye effluents containing recalcitrant residue into rivers and lakes.[5]Especially in textile industries produced more than 70% of the total quantity of waste in India.[6] Dyes are chemicals which bind to one of the materials then imparts colour due to presence of chromophore group. Industrially the following azo dyes are commonly used and they are acid dye, basic dye, direct dye, disperse dye, mordant dye, reactive dye and solvent dyes. The acid, basic, direct and reactive dyes are ionic.[7] Azo dyes are one of the largest and most versatile classes ofsynthetic dye. Azodyes consist of a diazotized amine coupled with an amine or phenol, and contain oneormoreazo linkages.Specifically reactive azo dye has complex aromatic structures. Mainly, those dyes arestable because they are very difficult to

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degrade.[8]These are mainly used in the textile,rubber products, paper printing, colourphotography, pharmaceuticals, cosmetics, foods, and other industries.

Over the past decades, biological decolorization has been investigated as a method to transform, degrade or mineralize azo dyes.[9] Moreover, such decolorization and degradation is an environmentally friendly and cost competitive alternative to chemical decomposition possess.[10] Unfortunately, most azo dyes are recalcitrant to aerobic degradation by bacterial cells.[11] However, there are few known microorganisms that have the ability to reductively cleave azo bonds under aerobic conditions.[2,12,13] Compared with chemical/physical methods, biological processes have received more interest because of their cost effectiveness, lower sludge production and environmental friendliness. Various wood-rotting fungi were able to decolorize azo dyes using peroxidases or laccases. But fungal treatment of effluents is usually time-consuming. Under static or anaerobic conditions, bacterial decolorization generally demonstrates good colour removal effects. However, aerobic treatment of azo dyes with bacteria usually achieved low efficiencies because oxygen is a more efficient electron acceptor than azo dyes.[14] With this scenario, present study was carried with the main aim to isolation and identification of dye degrading microorganisms.

Materials and Methods

Sample Collection

The effluent sample from textile industry located at Peenya Industrial Area, Bengaluru was collected in a clean sterile plastic container and taken to the laboratory for further analysis.

Isolation

Around 1 ml of effluent sample was aseptically added to 100 ml of LB media (Tryptone 10.0, Yeast 1.0, Agar 0.7, NaCl 10.0 & de-ionized water 1000ml) in 250 ml Erlenmeyer flask and incubated at 30ºC for 5 days on rotary shake at 150 rpm. The culture was serially diluted up to 10^{-6} dilution and the diluted cultures were spread plated aseptically and incubated at 30°C for 3 days.[15]

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Screening

1 gram of dye was dissolved in sterile distilled water and made up to 1000 mg/L. from the stock solution, required concentration of dye was prepared and filter sterilized using 0.5 µ membrane filter and used for further study. The selected strains must be screened to know the properly of degrading the dye, since they adapt to grow in the industrial effluent. The percentage of degradation observed by using UV-Spectrophotometer.

Assay of Dye Decolorization

Nutrient broth medium was prepared and 100 ml of the medium was dispensed in 250 ml of Erlenmeyer flasks. The medium was sterilized at 1 atmospheric pressure for 15 minutes. All the dyes (Blue and Red) were filtered and sterilized and added to the nutrient broth medium individually at a concentration level of 0.5g/L in an aseptic manner. To this, 1.0 ml of bacterial culture was inoculated and incubated at 37ºC for 24 hours on an orbital shaker. Control flasks containing without inoculum was also maintained. The samples were then analyzed for percent decolorization after incubation. The bacterial strains that exhibited maximum percent decolorization were selected for further studies.After incubation samples were filtered and centrifuged at 3500 rpm for 10 minutes and the suspended biomass was separated. The absorption spectra was measured at the lambda max of the dyes for the clear supernatant using a spectrophotometer. Medium containing dyes without the inoculum was taken as control. Percent decolorization was calculated with the following formula taking into consideration the initial and the final absorbance value of the dye.

Percent decolorization = (Initial absorbance – Final absorbance value x 100)/Initial absorbance values

Identification

Selected isolates were grown on nutrient agar plates. Based upon the growth characteristics,staining reactions and biochemical tests.[16]The isolateswere identified according to Bergey's Manual ofDeterminative bacteriology.[17]

Morphological Characterization

Macroscopic and Microscopic

Macroscopic analysis involved the observation of physical appearance of the incubated colonies *viz.* size, shape, color and texture of microorganisms by naked eyes. Microscopic observation involves the observation of shape (Bacilli, Cocci), size, color, and texture of bacteria fluorescent microscope after staining

Gram's staining reactivity

Morphological characterization of the potential isolates involved use of Gram's staining. The cells were studied based on their size, shape, arrangement and Gram's staining reactivity. A smear of the selected strains were prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat-fixed smear was flooded with crystal violet and after one minute, it was washed with water and flooded with mordant Gram's iodine. The smear was decolorized with 95 % ethyl alcohol, washed with water and then counter-stained with safranin for 45 seconds. After washing with water, the smear was dried with tissue paper and examined under oil immersion under microscope.

Motility test

The hanging Drop method, which involves collecting live microorganisms and removing them from a liquid medium, has been the most widely used method for studying cell movement and morphology. Using a ring of adhesive tape, circular concavities was made in a glass slide. vaseline was applied with a toothpick to the corners of the coverslip after placing a clean coverslip on its edges. In the middle of the coverslip, a loop of freshly made broth to test was transferred making sure to use a thin inoculum. To ensure that the vaseline is sealed within the concavity, the prepared glass slide or concavity slide upside-down (concavity

downwards) was placed over the drops on the coverslip. The slide was flipped so that it is on top. The organism was allowed for 1 minute to settle. The droplet was seen suspended across the concavity.

Biochemical Tests

Catalase test

Catalase is an enzyme that spilt up hydrogen peroxide into oxygen and water. Catalase is present in high concentration in majority of aerobic organisms, but absent in most obligate anaerobes. Heavy streaking of potential isolates was done on the surface of nutrient agar slant and incubated at 37 °C for 24 hours. 1 mL of hydrogen peroxide was then added over the growth on agar slant. The slide was observed for the rapid appearance and sustained production of gas bubbling.

Oxidase test

Heavy streaking of all potential isolates on surface of nutrient agar slant was done and incubated at 37 °C for 24 hours. Following incubation, a colony was picked up and a smear was made on a filter paper moistened with 1% tetraethyl phenylenediamine dihydrochloride solution. The formations of violet color within 45-60 seconds was observed.

Indole test

A loopful culture of selected potential isolates were inoculated in tryptone broth (1% Tryptone Water, 0.5 gm NaCl, 100 ml distilled water and pH 7.4) and incubated at 37°C for overnight. After incubation, 3-4 drops of xylene was added in medium and shaken vigorously. The two layers were allowed to separate and 1mL of Kovac's reagent was added slowly. The formation of pink color ring was observed.

Methyl red test

A loopful culture of selected potential isolates were inoculated in to glucose phosphate broth and incubated at 37°C for 48-72 hours. Following incubation, 5 drops of methyl red indicator was added in the medium. Development of red color was observed.

Voges Proskuer test

A loopful culture of selected potential isolates were inoculated in glucose phosphate broth and incubated at 37°C for 48-72 hours. Following incubation, 0.6 mL of α-naphthol and 0.2 mL KOH solution was added and shaken well. Development of cherry red colour was observed.

Citrate utilization test

Heavy streaking of all potential isolates was done on the surface of Simmon's citrate agar slant and incubates at slant position at 37°C for 48-72 hours. Development of deep blue colour within 24-48 hours was observed.

Nitrate utilization test

All potential isolates were inoculated in peptone nitrate broth and incubated at 37°C. 0.5 mL of α-naphthylamine reagent and sulphanilic acid reagent was added. Development of red colour was observed.

Christie–Atkins–Munch-Peterson (CAMP) test

A beta lysine producing strain, *Streptococcus aureus* was streaked down the center of a sheep blood agar plate. Incubated at 37°C for 18 to 24 hours. The streptococcal streak test organisms across the plate perpendicular to the *Streptococcus aureus* streak within 2 mm was observed for positive tests.

Urease test

Urease is a constitutively expressed enzyme that hydrolyzes urea to carbon dioxide and ammonia. A heavy inoculum from a 24hour pure culture was inoculated to the broth. The tube was gently shaken to suspend the bacteria. The tubes were incubated at 37°C for 24 to 48 hours. Observed the broth for a color change.

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Results and Discussion

Screening

The results of screening of bacterial isolates capable of dye decolorization was represented in table 1. Results depicted all the three isolates *viz.* IS 1, IS 2, and IS 3 shown maximum decolorization percentage at 150 mg/mL concentration. These findings depicted that decolourization was due to the metabolic activity of the organisms and not due to abiotic factor. Decolourization of synthetic dyes is the result of the cleavage of the chromophore group which generates colourless metabolic intermediates. The intermediate metabolite of the dye substrate is aromatic amines. The cleavage of the chromophore group of dyes is a reduction process which requires redox equivalents (electron donors) that transfer electrons to the chromophoric group (electron acceptors) of dye.[18,19]

Table 1: Screening of dye decolorization isolates

Bacterial Isolates	Dye	Dye Decolorization (%)				
		50 (mg/mL)	100 (mg/mL)	150 (mg/mL)	200 (mg/mL)	250 (mg/mL)
IS 1	Blue	56	64	69	43	31
	Red	35	43	48	22	10
IS 2	Blue	71	79	84	58	46
	Red	50	58	63	37	25
IS 3	Blue	23	31	36	21	12

	Red	15	23	28	13	4

In our study among the three isolates (IS 1, IS 2, and IS 3), IS 2 shown maximum decolorization potential of selected dyes at the concentration of 150 mg/mL, and hence IS 2 was selected for further morphological and biochemical characterization studies.

Identification and Morphological Characterization

Three isolates of bacteria capable of dye degration were identified in the screening. The shape of the bacteria (Bacilli, cocci), size, color, and texture of bacteria through fluorescent microscope after staining was observed. The pure colonies were observed under microscope by Gram staining technique (Table 2).

Table 1. Morphological characteristics

Bacterial Isolates	Colony Shape	Colony Colour	Margin	Elevation	Gram Staining	Motility test
IS 1	Bacilli	Cream	Regular	Raised	Positive	Non motile
IS 2	Cocci	Pale yellow	Regular	Raised	Negative	Motile
IS 3	Bacilli	Whitish	Entire	Convex	Positive	Non motile

Biochemical Characterization

The results of biochemical characterization of isolated strains were represented in Table 2. Results depicted that isolated strain (IS 2) was positive for catalase, oxidase, methyl red, Voges Proskauer, nitrate utilization, urease activity, casein hydrolysis, and starch hydrolysis tests. Whereas, the isolated strain (IS 2) was negative for indole, citrate utilization, and CAMP tests. The isolated bacterial strain (IS 2) was grown on *Bacillus cereus* agar plan, and hence IS 2 strain was identified as *Bacillus sp.*

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Table 2. Biochemical characteristics

S. No.	Biochemical Tests	Results
1	Catalase test	+ve
2	Oxidase test	+ve
3	Indole test	-ve
4	Methyl red test	+ve
5	Voges Proskauer test	+ve
6	Citrate utilization test	-ve
7	CAMP test	-ve
8	Nitrate utilization test	+ve
9	Urease activity	+ve
10	Casein hydrolysis	+ve
11	Starch hydrolysis test	+ve
12	Growth on selective medium	*Bacillus cereus*agar
13	Isolate	*Bacillus sp.*

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The Bacillus species isolated in this study decolourized the dye with chromophoric group (azo bond in golden yellow). This may be due to the source of effluent contaminated with textile dyes which contained various chromophoric groups. Members of the genus Bacillus have been reported to decolourize azo dyes.[20,21] The results of decolorization potential of isolated *Bacillus sp.* in our study was comparable with the findings of various other studies reported in the literature. Marimuthu et al., isolated the strains of *Bacillus sp.*, *Acinetobacter* and *Staphylococcus* and reported the decolourization ability of the isolated organism.[22]Mohan et al., isolated *Planococcus sp.* and *Bacillus sp.* from textile dye effluent and found that these organisms exhibited maximum decolourization which was about 80%.[23] Eisangela et al., have reported that *Staphylococcus* can decolourize azo dye in microaerophilic or aerated process in the presence of yeast extract.[24]

Conclusion

Today, bioremediation is a promising technology for cleaning up environmental contaminants because it is cost-efficient, easy to use, and environmentally friendly. The isolated and identified bacterial strains were discovered to be more potent at degrading textiles in a variety of environmental conditions. When textile dyes are being bioremediated, they make excellent bioagents. The newly isolated bacterial strain belongs to *Bacillus sp.* in our study was capable of azo dye decolorization. Hence newly isolated *Bacillus sp.*could be used as a good tool for bioremediation of various textile effluent treatment.

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