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Isolation of Dye Degrading *Bacillus* Species from the Soil near Dyeing Industry and Its Potential Application in Dye Effluent Treatment

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Abstract: The isolated dye degrading strain S1 showed Gram positive rod shaped motile bacteria. Biochemical tests made it confirmed that isolated dye degrading strain S1 belonging to genus *Bacillus* species on the basis respective results. The isolate fermented the sugars of glucose, sucrose, fructose, sorbitol while did not fermented lactose, xylose, arabinose and rhaminose. The results for gelatin hydrolysis, casein hydrolysis, oxidase test, catalase test, urease test, starch hydrolysis, nitrate reduction positive identification test profile for the dye degrading *Bacillus* species made it confirmed that isolated strains were belonging genus *Bacillus* species. The highest decolorizing activity were found at pH 7 (86.72%) and lowest decolorizing activity at pH 9 (64.34%). The effect of temperature for dye degrading strain *Bacillus* species S1 showed highest decolorizing activity at 30°C (89.36%) and lowest decolorizing activity at 45°C (70.34%). The *Bacillus* species S1 showed good ability to decolorize the dyes Red m 5B and T-Blue upto 300ppm. The dye degrading *Bacillus* species showed good ability to decolorize the dyes on metabolic shaker when compared to under static condition. Strain improvement for the selected isolate of dye degrading *Bacillus* species strain S1 showed that fewer colonies appeared on Petri plates with an increasing exposure to Ultra Violet light and similar result was observed with increasing concentration of Ethidium Bromide, the growth rate decreased as only few colonies appeared on the Petriplates.

Key words: Dye degradation · *Bacillus* species · Red m 5B and T-Blue · UV light · Ethidium Bromide

INTRODUCTION

Synthetic dyes are widely used in a number of industries, such as textile dyeing or printing, paper printing, cosmetics, pharmaceuticals and leather industries [1]. An increased demand for textile products, the waste waters generated from these industries have also increased proportionally and polluting the receiving water worldwide [2]. The organic dye stuffs, chrome dyes and other chemicals during various operations and produce a large quantity of solid and liquid waste containing hexavalent chromium [chromium (VI)], salts of zinc, sulphates, copper, sodium and potassium which causes severe toxicity to aquatic organisms [3]. The use of these water resources is limited and as well as an ecosystem is also affected by microbial populations and can be toxic, mutagenic and carcinogenic to animals [4]. The dye waste waters are becoming more and more complex with the increasing diversity of dye products. Thus, the treatment of the waste water is becoming top priority to the researchers [5]. More often these conventional modes of treatment lead to the formation of some harmful side products, an interest is therefore now focused on the microbial biodegradation of dyes as a better alternative as some microorganisms, including bacteria, fungi and algae, can degrade or absorb a wide range of dyes [6]. The biological mode of treatment for dye bath effluents offers distinct advantages over the conventional modes of treatment [7]. The best two dye degraders namely species of *Planococcus* and *Bacillus* were optimized for the effect of carbon and nitrogen source, pH, temperature and percentage of inoculum [8]. More than 50% of decolourization was achieved within four days of incubation and 80% decolorization after six days of incubation [9]. The isolates *Planococcus* species and *Bacillus* species exhibited maximum decolorization ability at pH between 5-8 and at temperature 37°C. Moreover, 10% (v/v) inoculums of glucose and peptone

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as carbon sources while nitrogen sources were found to be the optimum for decolourization [10]. Apparently the development of novel biological decolourization system consisting one or more acclimatized microorganisms in habitat concentration is urgently needed for the effective cleanup of the excess dyes in effluent. This study attempts to isolate and identify bacterial strains possessing strong dye decolourizing capacity, which can be potential candidate agent for the remediation of textile dye effluent

MATERIALS AND METHODS

Chemicals and Dye: The medium components used in this study were from Hi-media Labs, Mumbai (India) and other chemicals used were of analytical grade. The dyes used in this study are Red m 5B and T-Blue

Collection of Dye Waste Contaminated Soil Samples: Fifty soil samples were collected from waste disposal site of dye manufacturing plant and its surrounding areas [11].

Isolation and Screening of Bacteria from Soil Samples: Nutrient agar medium (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) was used for the isolation of bacteria from soil near waste disposal site of dye manufacturing plant and its surrounding areas. Serial dilutions from 10^{-1} to 10-7 were prepared by pipette out appropriate amount of distilled water suspension in 1 ml of dye contaminated soil sample. The nutrient agar plates were prepared and labeled. Then 0.1 ml of aliquot from 10^{-6} and 10^{-7} dilutions was pipette out into the corresponding nutrient agar containing Red m 5B and T-Blue (0.5% from that 15ml was added to 100ml of nutrient agar) plate. The sample was spread on the agar plate using the L-Rod (spread plate technique) and incubated at 37°C for 24hours [11]. After incubation, bacterial colonies were observed and results were interpreted.

Purification of Bacterial Colonies: Each morphologically distinct colony appeared on Petri plate was further purified into single colony by repeated streaking on Petri plates containing nutrient agar media. After incubation at 37°C for 48 hours, plates were observed for single bacterial colony. All colonies were isolated, sub-cultured and identified by several morphological observation by Gram's staining, motility test, biochemical tests (indole, methyl red, voges-proskauers, citrate, triple sugar iron), carbohydrate fermentation tests (glucose, lactose, fructose, xylose, arabinose, sorbitol, rhaminose) and enzymatic test (starch hydrolysis, gelatin hydrolysis, casein hydrolysis, urease, catalase, oxidase, nitrate reduction tests) by following methodology of Holt *et al*. [12].

Determination of Effluent Decolorization: Determination of effluent decolorization by the bacteria was followed by methodology of Anjali *et al.* [13]. Different aliquots of textile effluent were taken in Erlenmeyer flasks (20, 40, 60 and 80 ml) in triplicates and 2.5 ml of 12 hours old *Bacillus* species culture was added in each Erlenmeyer flask (cotton plugged and labeled). All the Erlenmeyer flasks were mixed thoroughly and incubated at room temperature for decolourization from 12 hours to 48 hours. During the incubation period, samples were drawn at different time intervals of 12 hours, 24 hours and 48 hours, samples were then centrifuged at 5000 rpm at 4°C for 10 minutes using cooling centrifuge (DVCI-S122 Remi Instruments Mumbai). The decolourization potential of the isolate was determined by taking the absorbance reading of the cell free supernatant using Spectrophotometer at 700nm. The extent of decolourization was expressed as percent (%) decolourization and estimated as (Ai - At)/ (At x x100) where initial absorbance of the dye solution and absorbance at cultivation time denoted by Ai and At respectively.

Preparation of Dye Stock and Working Concentration: In this study two dye samples were collected from commercial dye manufacturing Industries namely Red m 5B and T-Blue. They were used at different concentrations to amend culture media. A stock (0.5g /l00ml) from each dye was prepared in sterile water by adopting methodology of Aftab *et al*. [14].

Decolorization Ability of Bacteria in Liquid Media: Decolorization ability of bacteria in liquid media was followed by methodology of Anjali *et al.* [13]. Decolorization of dye samples was studied in nutrient broth medium having 100ppm concentration of dyes. 0.25ml of seed culture was inoculated to 4.75ml of dye amended nutrient broth and kept for incubation for 72 hours at room temperature on metabolic shaker. Three controls were maintained without addition of seed. After 72 hours of incubation all samples were centrifuged at 10,000 rpm for 10 minutes and absorbance value was taken by UV-Vis spectrophotometer at 700nm wavelength according to the dyes.

Effect of Physical and Chemical Parameters like pH, Temperature, Dye Concentration and Agitation on Dye Degrading Ability: The physical and chemical parameters are carried out by following the methodology of Ong *et al*. [15] and Dawkar *et al*. [16].

Effect of pH: Dye degrading *Bacillus* species strain S1 was inoculated to above different pH (pH 6, pH 7, pH 8 and pH 9) nutrient broth media and 5% of its seed culture was added to the dye amended different pH media. After incubation for 96 hours, effect of pH was compared.

Effect of Temperature: Dye nutrient broth was inoculated with seed culture dye degrading *Bacillus* species strain S1 was incubated for 48 hours at different temperatures of 30°C, 37°C, 40°C and 45°C. The effect of different temperatures was compared after the incubation for 96 hrs.

Effect of Dye Concentration: In this study Red m 5B and T-Blue two dye concentrations of 100ppm (50µl stock dye into 5ml nutrient broth), 200ppm (100µl stock dye into 5ml nutrient broth), 300ppm (150µl stock dye into 5ml nutrient broth) and 400ppm (200µl stock dye into 5ml nutrient broth) and 5% seed culture of Bacillus species strain S1 was added to each concentration after which they were incubated for 48 hours after which the effect was compared using spectrophotometer.

Study of Effect of Agitation: In these study two sets of triplicates of 100ppm dye concentrations of Red m 5B and T-Blue separately were prepared and inoculated with 5% of seed culture. One set was kept on shaker (100 rpm) and other set was kept under static condition. After 96 hours of incubation effect was compared using UV-spectrophotometer.

Strain Improvement of Selected Isolate Using UV and Ethidium Bromide: The dye degrading *Bacillus* species strain S1 was grown for 24 hours in LB broth and exposed to UV light at interval of 5 minutes to induce mutagenesis. And also dye degrading *Bacillus* species strain S1 was grown for 24 hours in LB broth prior to being treated with different concentration of EtBr for 60 minutes to induce mutagenesis [17].

RESULTS

Isolation and Screening of Bacteria from Soil Samples: Different bacteria were isolated from the soil samples which were collected from the dye contaminated sites. Some bacteria have got natural ability to degrade dyes by continuous exposure to dye polluted environment. The dye degrading bacterial strain S1 shown a zone around the colonies in the medium containing T-Blue (Fig. 1) and in the medium containing Red m 5B (Fig. 2) respectively.

Purification and Identification of Bacterial Colonies: Bacterial colonies obtained by isolation were purified by streaking on Petri plates having nutrient agar media with dye degrading *Bacillus* species strain S1. A single pure colony was obtained and identified by morphological and biochemical characterizations are shown in Table 1 and Table 2.

Determination of Effluent Decolorization: Decolorization of different concentrations (20, 40, 60 and 80 ml) of aliquots textile dye effluent after treatment of degrading strain S1 *Bacillus* species are represented in the Table 3. The dye degrading strains showed good ability to degrade the dye after incubation at various intervals of time for 12 hours, 24 hours and 48 hours.

Preparation of Dye Stock and Working Concentration: In this study two dye samples were collected from commercial dye manufacturing Industries namely Red m 5B and T-Blue. They were used at different concentrations to amend culture media. A stock (0.5g/l00ml) from each dye was prepared in sterile water.

Decolourization Ability of Bacteria in Liquid Media: Decolourization ability of bacteria *Bacillus* species dye degrading strain S1 in liquid media of in nutrient broth medium having 100ppm concentration of dyes Red m5B and T-Blue an absorbance value was taken by UV-Vis spectrophotometer at different wavelengths according to the dyes are presented in Table 4.

Effect of Physical Parameters like pH, Temperature, Dye Concentration and Agitation on Dye Degrading Ability

Effect of pH: After incubation for 96 hours, effect of pH was compared for the dye degrading strain *Bacillus* species S1 which displayed the highest decolorizing activity at pH 7 (86.72%), pH 6 (84.36%), pH 8, (78.57%) and lowest decolorizing activity at pH 9 (64.34%).

Effect of Temperature: After incubation for 96 hours, effect of temperature was compared for the dye degrading strain *Bacillus* species S1 which showed the highest decolorizing activity at 30°C (89.36%), 37°C (80.12%), 40°C (73.10%) and lowest decolorizing activity at 45°C (70.34%).

Table 1: Identification test for morphological and biochemical characters of dye degrading strain S1

Test	*Bacillus* species	Test	*Bacillus* species
Gram staining	Gram Positive rods	Glucose	Fermented
Flagella staining	Absent	Lactose	Non-fermented
Motility test	Motile	Sucrose	Fermented
Indole	Negative	Fructose	Fermented
Methyl Red	Negative	Xylose	Non Fermented
Voges Proskaure	Negative	Arabinose	Non-fermented
Citrate	Negative	Soribitol	Fermented
Triple Sugar Iron	$A/AK^{G\text{-}ive}$	Rhaminose	Non Fermented

Table 2: Enzyme secreting identification characters of dye degrading strain S1

S.NO	Test	*Bacillus* species
1	Gelatin hydrolysis	Zone of Hydrolysis
2	Casein hydrolysis	Zone of Hydrolysis
3	Oxidase test	Positive
4	Catalase test	Positive
5	Urease test	Negative
6	Starch hydrolysis	Zone of Hydrolysis
7	Nitrate reduction	Positive

Table 3: Decolorization of textile dye effluent after treatment of *Bacillus* species dye degrading strain S1

Dye Concentration	Incubation for 12 Hours	Incubation for 24 Hours	Incubation for 48 Hours
20ml	76.42±0.35	82.29±0.57	96.22±0.27
40ml	71.33±0.75	75.09±0.69	94.82±0.44
60ml	68.82±0.29	72.27±0.20	95.19±0.21
80ml	66.42±0.19	70.34±0.40	97.62±0.62

Table 4: Dye decolorization in a liquid medium after treatment of dye degrading strain S1 *Bacillus* species

Dye used	Time	*Bacillus* species
Red m5B	0	1.049
	48	0.375
	96	0.175
T-Blue	0	0.910
	48	0.610
	96	0.153

Fig. 1: Showed the dye degrading colonies including *Bacillus* species S_1 Strain in the nutrient agar containing T-Blue (0.5% from that 15ml was added to 100ml of Nutrient agar)

Effect of Dye Concentration: After incubation of 48 hours the dye degrading *Bacillus* species S1 in the four dye concentrations of 100ppm, 200ppm, 300ppm and 400ppm while compared showed good ability to decolorize the dyes Red m 5B and T-Blue in 300ppm.

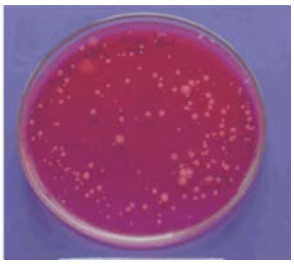

Fig. 2: Showed the dye degrading colonies including *Bacillus* species strain S_1 in the nutrient agar madium containing Red m 5B (0.5% from that 15ml was added to 100ml of Nutrient agar madium)

Study of Effect of Agitation: After 96 hours of incubation effect was compared by using UV-spectrophotometer and was found that 100ppm dye concentration were inoculated with 5% of seed culture of dye degrading *Bacillus* species strain S1 which showed good ability to decolorize the dyes on metabolic shaker when compared to under static condition.

Strain Improvement of Selected Isolate Using Uv And Ethidium Bromide: Strain improvement of the selected isolate of dye degrading *Bacillus* species strain S1 was done and it was observed that fewer colonies appeared on Petri plates with increasing exposure to UV light.

Similar result was observed with increasing concentration of Ethidium Bromide (EtBr) treatment with cultures. So, it can be concluded that colonies appearing in Petri plates with higher concentration of EtBr treatment and long exposure to UV light can easily survive in environment.

DISCUSSION

Remediation of dyeing industry effluent by using microorganisms has proved to be the best solution since numerous bacterial species including *Bacillus, Pseudomonas, Enterobacter, Halobacter* and *Aeromona*s have been reported to exhibit tremendous capability to decolourize and detoxify a wide range of azo dyes composed of phenylamine, benzenediazonium chloride or phenol [18, 19]. In the present study bacterial strains were isolated from textile dye effluent on the basis of morphological, biochemical, carbohydrate and enzyme characteristics, it was identified as *Bacillus* species by comparing with previous study [20, 21]. Bioremediation is widely used to clean up both soil and waste water containing organic and inorganic contaminants [22]. In our study we have isolated the *Bacillus* species by comparing with previous report of the bacterial species including *Bacillus subtilis, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and *Escherichia coli* were had been isolated from the textile dye effluent [23]. It has also been reported that three different bacterial species, Bacillus species *Escherichia coli* and *Pseudomonas fluorescens* were isolated from textile dye effluent contaminated soil and used for the degradation of dye [24, 25]. The bacterial isolates, *Bacillus* species their ability was measured and found to have significant potential to decolorize the dye effluent by comparing with previous study [26]. Our study showed that *Bacillus* species can be used a potential microbe in the biodegradation of azo dyes namely Red m B5 and T-Blue. These microbial consortia were recommended for environmental remediation to degrade variety of pollutants. Therefore, this study was aimed to evaluate the ability of microbial isolates *Bacillus* species in decolourization of dye-effluent generated from local textile industry [27]. The literature revealed that highest decolourization percentage of Coractive Blue 3Rdye effectively during optimization and more interestingly consistent decolourization of textile dye [28, 29]. Decolourization was effective at pH 8 and at 35°C with starch, peptone as carbon and nitrogen sources in static conditions [30]. The treatment systems composed of mixed microbial populations of six different bacterial isolates *viz*., *Bacillus odyssey, Bacillus thuringiensis, Bacillus subtilis, Bacillus cereus, Alcaligenes* species and *Nocardiopsis alba* achieve a higher degree of biodegradation and mineralization due to the synergistic metabolic activities of the microbial community and have considerable advantages over the use of pure cultures in the degradation of synthetic azo dyes [31, 32]. *Bacillus* species isolated from soil contaminated with untreated textile mill effluent utilized an Azo dye as sole source of carbon and nitrogen [33]. Decolorization of azo dye Red 3BN by two bacterial species *Bacillus cereus* and *Bacillus megaterium* has been analyzed using mineral effluent, consisting of known concentration of the dye in medium [34]. The Physical and chemical parameters like temperature, agitation, pH and dye concentration after optimization showed highest decolourization variation by changing one parameter to another at a time supporting the previous literature [35]. In the present study *Bacillus* species Showed 97% decolourization ability, the reason for effective and faster decolourization of the effluent by bacteria might be associated with the metabolic activities and interactions of these strains while comparing with previous literature [36]. In this study, the reduction of pollution parameters indicates the fact that the process of bioremediation carried out by *Bacillus* species would be a potential microbe for biodegradation of azo dyes in the industrial effluent by comparing with previous literature [20].

CONCLUSIONS

This work was carried out to screen, characterize tentatively identify the isolated bacteria from soil sample with best decolorizing ability. In this study bacteria were isolated from soil samples collected from waste disposal sites of dye and pigment industry and its surrounding areas. All isolates were investigated for their dye-degrading activity out of which seven isolates were found to have dye decolorizing activity with different efficiency. Out of twenty different bacterial isolates found in fifty soil samples, best dye-degrading activity was found in dye degrading strain S1. All studies were carried out in triplicates and their average was taken as final reading. An attempt was made to characterize the bacteria based on morphological, biochemical and enzymatic properties. The best strain was selected and identified to be as dye degrading *Bacillus* species S1 according to identification flow charts of Bergey's Manual of Determinative Bacteriology. Dumping of dye stuff and dye waste water into the environment results in

environmental pollution and medical problems. There is an urgent need for simple and cost-effective treatment methods for this problem. Microbial degradation and decolorization of the dyes gives us a hope to solve this problem as it is environment friendly, cost-effective and produces no harmful intermediates. Bacteria have an advantage over other microbes as they have a high growth rate and a high hydraulic retention time. Since various physicochemical parameters influence the decolorization performance, optimization of these is essential. Also further, to ensure the safety of the decolorized wastewater, studies should be conducted on the toxicity of the treated dye solution.

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