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Isolation of coomassie brilliant blue-G dye degrading bacteria from effluent, screening & evaluation of their dye decolorization activity

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Abstract
Rapid industrialization has led to the accumulation of various toxic elements that harm the environment and ultimately affect human lives. Synthetic dyes are widely used because of cost-effective synthesis, high stability to light, temperature, etc. as compared to natural dyes. Textile dye effluents generally consist of toxic synthetic dyes which consist of 50%-70% of azo dyes. Bioremediation has become a key to dealing with hazardous pollutants. Various bacteria such as *Bacillus subtilis*, *Aeromonas hydrophila*, and *Bacillus cereus*, fungi & actinomycetes have been found to possess dye decolorizing activity. Bacteria from dye-contaminated effluent may possess dye decolorizing ability due to their adaptation to extreme environmental conditions. Media consisting of Coomassie brilliant blue-G at different concentrations ranging from 0.005%, 0.01%, and 1 % were used to isolate dye degrading organisms from effluent samples. Colony characteristics of organisms obtained from medium containing Coomassie brilliant blue-G were studied and biochemical tests i.e., IMViC, TSI were performed for identification. Dye decolorization assay was performed colorimetrically at 615 nm for concentrations of Coomassie brilliant blue-G ranging from 0.005%, 0.01%, and 1% every 24 hours. The percentage of dye decolorization activity was found to be 94%, 28%, and 0.07% for Coomassie brilliant blue-G for concentrations ranging from 0.005%, 0.01%, and 1% respectively. The probable organism isolated that helped in decolorization of 0.005% concentration may be of Gram-positive species, for 0.01% concentration may be *Pantoea agglomerans* and for 1% may be *Escherichia sp*. The probable isolates obtained need to be further investigated regarding various factors such as media composition affecting dye degradation & mechanism of dye degrading activity.

Keywords: synthetic dyes, bioremediation, Dye decolorization assay, *pantoea agglomerans*, *escherichia sp.*, coomassie brilliant blue-G

Introduction

Rapid industrialization has led to the accumulation of various toxic elements that harm the environment and ultimately affect human lives. Industrial processing often leads to effluents that consist of waste materials and can be toxic not only to humans but also to aquatic life. These waste products can also accumulate in the soil and lead to toxicity. Every industry must have its wastewater treatment plant. But most of the industries lack this and thus wastewater is directly let out into water bodies without any treatment [1].

Textile dye effluents generally consist of toxic dyes and heavy metals. In the ancient age, natural sources were used for drying clothes. But the extraction process was a bit difficult and also expensive. Hence there was a need for synthetic dyes. In 1856, English chemist William Henry Perkin, in his experiment with aniline (one of the simplest chemical components of coal tar) obtained a black precipitate and discovered purple color, which readily dyed silk and was much more stable in sunlight than any other (natural) purple dye than in use. [1] According to the annual report of the Union Ministry of Environment and Forest, some 4.4 million tonnes of hazardous wastes are being generated by 13,011 units spread over 373 districts of India [2].

Dyes are natural or synthetic colored organic compounds having the property of imparting their color to other substances, such as textile fibers [1]. Synthetic dyes are used extensively for textile dyeing, paper printing, leather dyeing, color photography and as additives in petroleum products because of their ease and cost-effectiveness in synthesis, firmness, high stability to light, temperature, detergent, and microbial attack, and variety in color as compared to natural dyes [3]. Approximately, 10,000 different dyes and pigments are used in different industries and their production exceeds over 7×10^5 tones annually worldwide [4].

Textile industries effluents generally consist of 0.6–0.8 g^{L-1}. [5] Various methods can be used for the removal of the dyes from the wastewater. [6] These methods include physical, chemical, and biological methods that help in decolorization. The physical method generally includes coagulation-flocculation but is less efficient in the dye removal process from wastewater. Also, it tends to produce high amounts of sludge [7].

The adsorption method includes using an adsorbent that can adsorb the dye molecules based on an affinity in the wastewater.[8] Activated carbon acts as the best adsorbent but is expensive.[9] Another low-cost substitute can be used such as wheat straws, maize stalks, peat, clay, etc but regeneration and disposal are tediously associated with problems such as high sludge production [11, 12]. filtration methods such as nanofiltration, ultrafiltration, reverse osmosis can be used efficiently for dye removal but have other limitations such as the high cost of membranes, membrane fouling, etc [12]. Chemical methods generally use oxidizing agents such as ozone (o_3), hydrogen peroxide (H_2O_2), and permanganate (MnO_4) but have limitations such as high cost, low COD removal capacity, etc [6]. Thus, physical and chemical methods are either costly or are inefficient incomplete dye removal or tend to produce high sludge, etc. To overcome these issues biological methods can be used such as "Bioremediation" [6].

Bioremediation is an efficient tool in dealing with pollutants and is widely used in environmental sciences [13]. Microorganisms are used for complete degradation or mineralization of dyes in wastewater and have certain advantages over physical and chemical methods such as a) eco-friendly b) low-cost c) less sludge production d) nontoxic end products etc [14, 15]. Various microorganisms including, yeasts, *Proteus sp.*, *Enterococcus sp.*, *Streptococcus sp.*, *Bacillus subtillis*, and *Streptococcus sp.* have been previously isolated to degrade azo compounds [16]*Sphingomonas xenophaga* BN6, *Agrobacterium tumefaciens*, *Ralstonia eutropha* 335, *Hydrogenophaga palleronii*, *Escherichia coli* K12, and *Flexibacter filiformis* (Gram-negative), *Bacillus subtilis*, *Rhodococcus erythropolis*, and *Lactobacillus Plantarum*) (Gram-negative) and Archea (*Halobacterium salinarum*) can degrade azo dyes under anaerobic conditions.[1] Bacteria are widely used as many of them possess the ability to degrade the azo dyes and eliminate them through wastewater and the environment via various mechanisms such as biosorption, bioaccumulation, oxidative process, reductive process, sequential oxidative-reductive process, etc [17].

Bacterial enzymes responsible for the degradation of azo dyes

Nowadays most industries rely on enzymatic technology to treat dye contaminated wastewater. Two kinds of enzymes lead to decolorization *viz*. reductive enzymes and oxidative enzymes [17].

Reductive enzymes

Flavin-dependent and nonflavin reductases are two broad types of reductive enzymes. Flavin-dependent azoreducatses, there are two cycles where NADPH-dependent reduction of FMN to FMNH takes place. In the first step azo dye gets converted to hydrazine and in the second step, hydrazine gets converted to two constitutive amines. The flavin-dependent azoreductases are considered to be polymeric and also provide thermal stability to the azoreductase enzyme [17].

Azoreductases can be classified into two groups depending on the electron donor requirement *viz* a) flavin-containing that relies on NADH and b) relies on NADPH as a reductant for decolorization of dye. Burger & Stolz (2010) isolated the first flavin-free azoreductase enzyme from Xenophilus azovorans KF46F strain and was oxygen tolerant. The mechanism of flavin-free azoreductase is different as compared to flavin-dependent as they rely on NADPH as a reductant.

NADH-DCIP and riboflavin reductase also plays the role in dye decolorization but are not widely accepted as they have limited application as they are infective *in vivo* [17].

Oxidative enzymes

Oxidative enzymes such as lignin peroxidase, laccase, and tyrosinase play a major role in dye decolorization activity. Peroxidase substrates are been degraded by peroxidase enzymes. Lignin peroxidase and very alcohol oxidase in combinations increase the efficiency of dye degradation activity of azo and anthraquinone dyes. Also, mono-rhamnolipid-like molecules increased the activity of lignin peroxidase and retail alcohol which may be as these molecules may provide protection from inactivation of the enzyme by hydrogen peroxide or any other factor. Telke *et al*. (2009) reported a novel enzyme, a laccase-like phenol oxidase that can react with non-phenolic substrates [17]. Laccases are oxidoreductase that catalyzes the dye decolorization process by direct oxidation or by indirect oxidation by utilization of mediators that help accelerate the reaction process. These laccase mediators are considered to be good substrates as they can be stable even in an oxidized and reduced state, also they possess no inhibitory effect on enzyme activity. Laccases that are produced by *streptomyces* are reported to be effective in dye decolorization [17].

Oxidative enzymes are also produced by fungi and can also be used in dye decolorization. But the oxidative enzymes produced by bacteria are reported to be more stable as compared to fungal oxidative enzymes [17].

Materials and Methods

The study was conducted at the Department of Biotechnology, Smt. C.H.M College, Ulhasnagar-03.

1. Sample Collection [28]

Textile effluents are directly released into the Ulhas River. Thus, the effluent sample was collected from Ulhas River, Ulhasnagar East in airtight bottles and was filtered through filter paper to remove large suspended

particles and the filtrate was used for the isolation procedure. Nutrient agar was used for isolation. Glucose phosphate broth, Simmon's citrate agar, Tryptone broth, Triple sugar ion agar were used for performing biochemical tests.

2. Physico-chemical property analysis [29].
The collected effluent samples have been analyzed to determine their physicochemical parameters. The various parameters like Chemical oxygen demand (COD), Biological oxygen demand (BOD), were analyzed in the laboratory by the standard protocol [29].

3. Enrichment and isolation of dye tolerating strains
a) Primary screening:
Enrichment [28].
The sample collected was subjected to enrichment culture technique.
Enrichment was carried out in 3 different flasks containing 100ml Sterile Nutrient Broth with 1% Coomassie brilliant blue-G, for 1 week. A loopful from the same was streaked on sterile nutrient agar plates containing 1% Coomassie brilliant blue-G. Similarly, a loopful of the effluent sample was directly streaked on sterile nutrient agar plates containing 0.005% and 0.01% of Coomassie brilliant blue-G. All the plates were incubated at room temperature/72hours.

b) Secondary Screening of dye decolorizing strains [28]
The isolated colonies obtained from the above plates were again streaked on sterile nutrient agar plates containing 0.005%, 0.01%, and 1% Coomassie brilliant blue-G. Plates were incubated at room temperature. The isolated organism was streaked on sterile nutrient agar slants containing 0.005%, 0.01%, and 1% Coomassie brilliant blue-G and was preserved at 4 ^{0}C until further use.

Dye Decolorization Assay [28]
5ml of saline suspension of the isolated organisms was inoculated in 1oo ml sterile nutrient broth flask containing 0.005%, 0.01%, and 1% of Coomassie brilliant blue-G was incubated at room temperature for 10 days. 5ml was withdrawn every 24 hours and centrifuged at 2500 rpm and was subjected to a colorimeter to read optical density at 615nm.

Percentage of dye decolorized was calculated by following formula [28]

Percentage of Dye Decolorization = Initial O.D – Final O.D / Initial O.D x 100

Results
Textile effluents are directly released into the Ulhas River at Ulhasnagar. Thus, only one effluent sample from Ulhas River, Ulhasnagar East was used.
Isolation of organisms from the effluent sample on Nutrient agar with respective dye concentration as follows:-

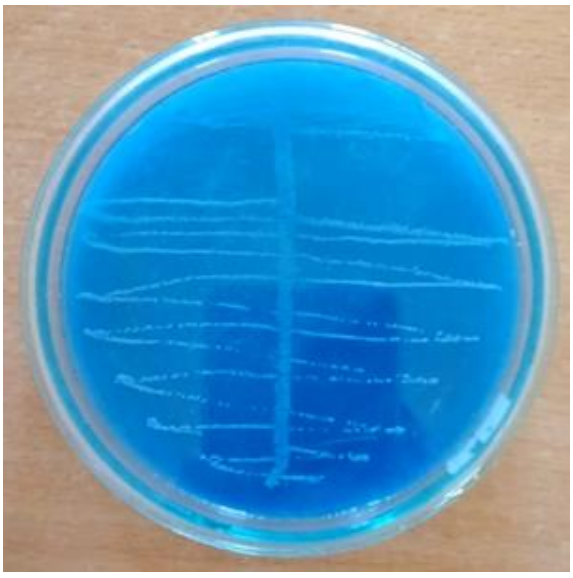

Fig 1: Growth obtained on sterile nutrient agar plate containing 0.005% Coomassie brilliant blue-G

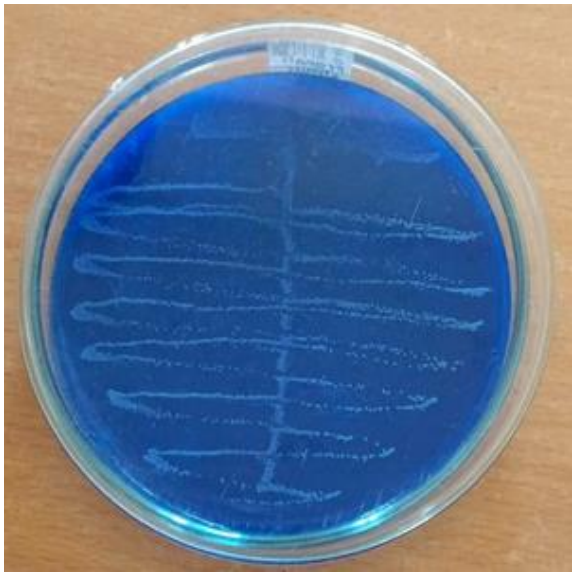

Fig 2: Growth obtained on sterile nutrient agar plate containing 0.01% Coomassie brilliant blue-G

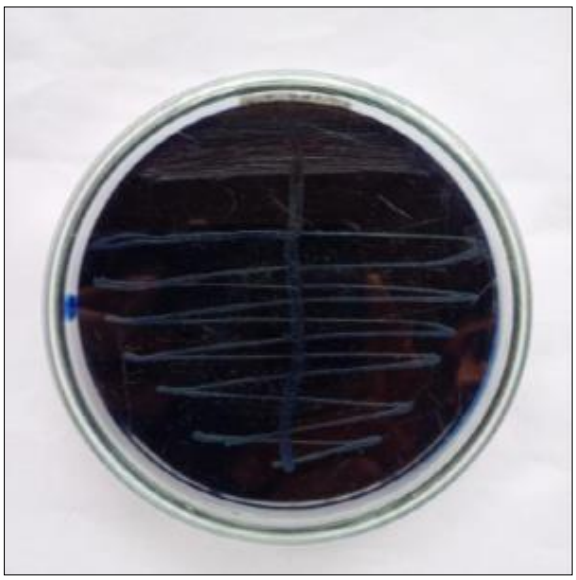

Fig 3: Growth obtained on sterile nutrient agar plate containing 1% Coomassie brilliant blue-G

Biochemical tests – IMViC and TSI test

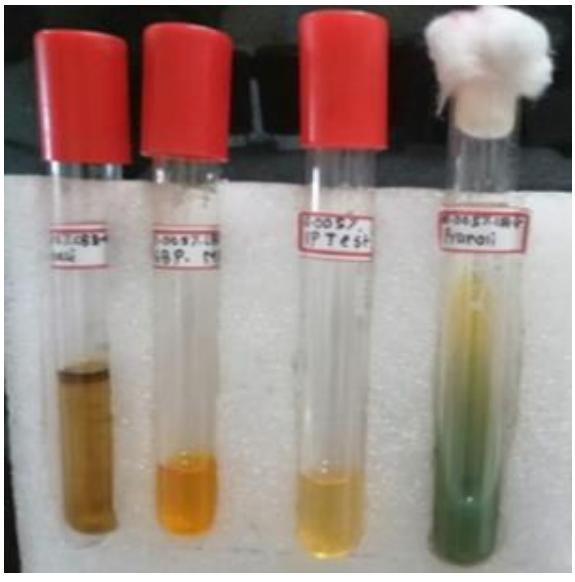

Fig 4: IMViC test for identification of isolates obtained on plate with 0.005% Coomassie brilliant blue-G.

Fig 5: IMViC test for identification of isolates obtained on plate with 0.01% Coomassie brilliant blue-G.

Fig 6: IMViC test for identification of isolates obtained on plate with 1% Coomassie brilliant blue-G.

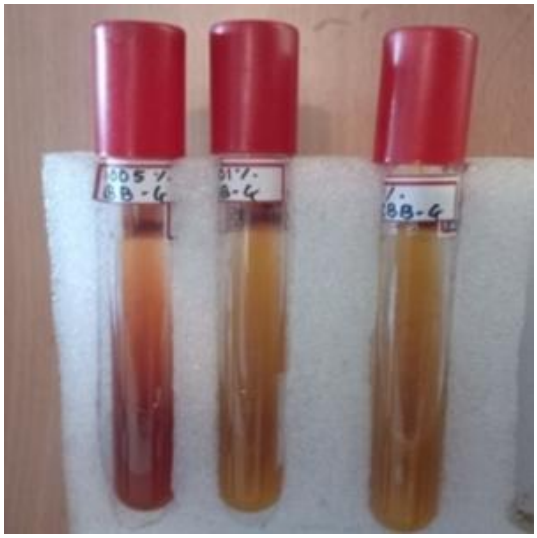

Fig 7: TSI test for identification of isolates obtained on plate with 0.005%, 0.01% and 1% Coomassie brilliant blue-G respectively.

Table 1: Prediction of the probable organism isolated on sterile nutrient agar plates containing 0.005%, 0.01%, and 1% Coomassie brilliant blue-G concentration based on IMViC and TSI test.

Sr. No	Media with respective concentrations of dyes	Probable organism isolated
1.	Nutrient Agar + 0.005% Coomassie brilliant blue-G	-
2.	Nutrient Agar + 0.01% Coomassie brilliant blue-G	*Pantoea agglomerans*
3.	Nutrient Agar + 1% Coomassie brilliant blue-G	*Escherichia coli*

Dye decolorization assay

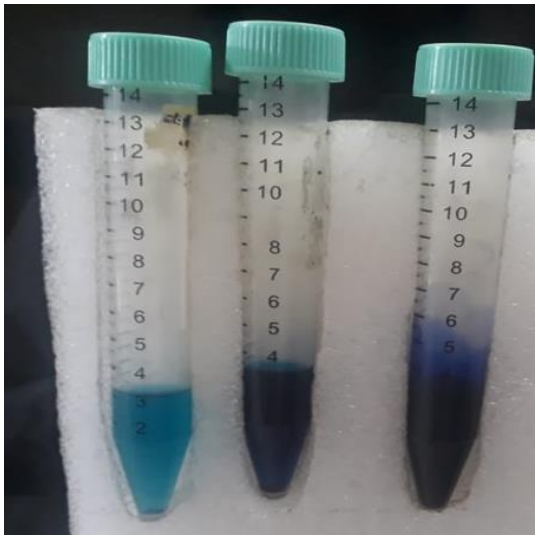

Fig 8: Day 0 - 0.005%, 0.01% and 1% Coomassie brilliant blue-G respectively.

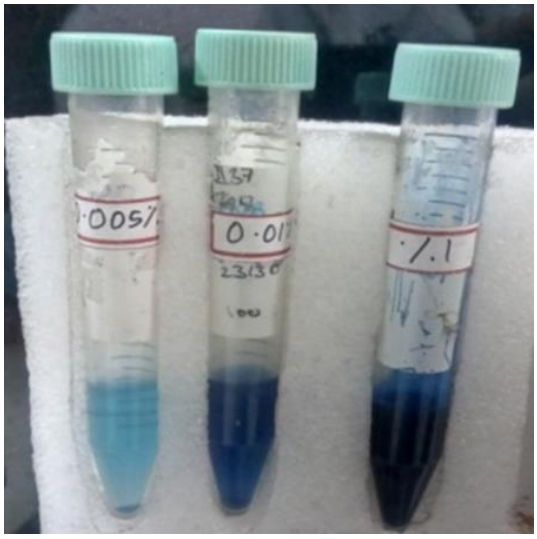

Fig 9: Day 10 - 0.005%, 0.01% and 1% Coomassie brilliant blue-G respectively.

Table 2: Dye decolorization assay results

Sr.no	No of Days	O.D 615 nm		
		0.005% Coomassie brilliant blue-G	0.01% Coomassie brilliant blue-G	1% Coomassie brilliant blue-G
1.	Day 0	>1.0	>1.0	>1.0
2.	Day1	>1.0	>1.0	>1.0
3.	Day2	>1.0	>1.0	>1.0
4.	Day3	0.91	>1.0	>1.0
5.	Day4	0.80	>1.0	>1.0
6.	Day5	0.69	>1.0	>1.0
7.	Day6	0.52	>1.0	>1.0
8.	Day7	0.41	>1.0	>1.0
9.	Day8	0.29	>1.0	>1.0
10.	Day9	0.11	>1.0	>1.0

11.	Day10	0.07	>1.0	>1.0
Percentage of Dye Decolorization		94%	28%	0.07%

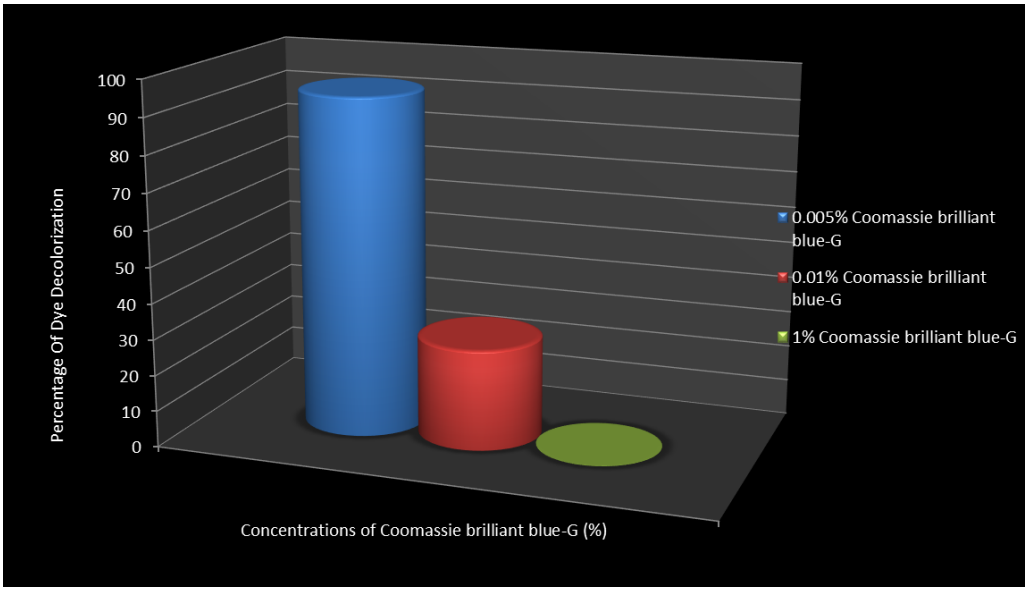

Chart 1: Graphical Representation of Dye Decolorization Assay for 0.005%, 0.01% and 1% concentration of Coomassie brilliant blue-G (615nm)

Discussion

Dye contaminated wastewaters are not only harmful to aquatic life and the environment but are also harmful to human life. Bioremediation can be used as an effective tool to deal with such dye-contaminated effluents released from textile industries. By performing Gram's Staining, the Gram nature of all the isolated organisms was found to be Gram-negative coccobacilli. The probable organism isolated that helped in decolorization of 0.005% concentration may be of Gram-positive species, for 0.01% concentration may be *Pantoea agglomerans* and for 1% may be *Escherichia sp*.

The percentage of dye decolorization activity was found to be 94%, 28%, and 0.07% for Coomassie brilliant blue-G for concentrations ranging from 0.005%, 0.01%, and 1% respectively. Degradation of Coomassie brilliant blue-G dye at 0.01% and 1% concentrations started late as compared to 0.005% concentration. As O.D was obtained > 1.0 still there was a decrease in color intensity observed. A control flask was kept, which contained only nutrient broth & respective concentrations of dye. It was observed that the dye intensity & O.D was constant throughout the incubation period is Day 0 to day 10. This indicates that the dye is not getting degraded spontaneously but is getting degraded due to microorganisms in the medium.

A study conducted by Alyssa M. Walterson and John Stavrinides reveals that the bacterial genus Pantoea was discovered about 25 years ago but approximately 20 species are yet known. Isolates that are been obtained from water and soil are mostly used in the biodegradation and bioremediation of toxic products released from industries into the environment [18]. A study conducted by Moutaouakkil A, Zeroual Y, Zohra Dzayri F, Talbi M, Lee K, Blaghen M reveals that *Pantoea agglomerans* that was isolated from dye contaminated sludge was found to possess 28,000 Da aerobic azoreductase enzyme. The enzymatic activity was found to be NADH dependent and the enzyme was monomeric flavin-free azoreductase. It helped in the degradation of dyes such as Methyl Red, Disperse Yellow, Trypan Blue, Amaranth, and Orange G [19]. A study was conducted by Adnane Moutaouakkil, Youssef Zeroual, Fatima Zohra Dzayri, Mohamed Talbi, Kangmin Lee, Mohamed Blaghen in which *Pantoea agglomerans* was immobilized in a fluidized bed bioreactor on different supports to decolorize the azo dye i.e. Methyl red at various concentrations. The fluidized bed bioreactor was prepared in a 500 ml flask which consisted of 100 ml of Minimal Media with 0.1% (wt/vol) of glucose and 100 mg/L of Methyl red and cells immobilized in different supports. The bioreactor was then placed on a rotary shaker at 100 rpm (25^{oC}). For immobilization of cells calcium alginate, polyacrylamide, copper beech, vermiculite, etc was used and the rate of decolorization was found to be higher in cells that were immobilized in polyacrylamide as compared to vermiculate and cooper [20]. But calcium alginate provides greater stability and shows higher purifying capability. [20]

In a study conducted by Balraj Bandary, Zakir Hussain, Rakesh Kumar the effect of carbon and nitrogen sources on *Escherichia coli* bacteria in removing dyes such as Methylene blue and Methyl orange was determined. The results show that Glucose and ammonium sulfate are the best sources of carbon and nitrogen [23].

In a study by Nakanishi M, Yatome C, Ishida N, Kitade Y an FMN-dependent NADH-azoreductase was identified and partially characterized from *Escherichia coli.* The *Escherichia coli* azoreductase has been identified in Enterococcus faecalis and was found to have 34% similarity [24].

In a study by Chen H, Wang RF, Cerniglia CE the azoreductase enzyme produced was the enzyme was not only able to decolorize Methyl Red, but was also able to convert the sulfonated azo dyes Orange II, Amaranth, Ponceau BS, and Ponceau S [25].

The organisms isolated need to be further investigated regarding various factors such as media composition affecting dye degradation & mechanism of dye degrading activity. The azoreductase enzymes produced by the organisms need to be studied, identified and evaluation of it is required. Also, various conditions that lead to higher biomass concentration need to be evaluated. The products that are produced after the mineralization of dyes needs to be evaluated on basis of toxicity. All the necessary factors that affect the rate of dye decolorization activity need to be considered. Industrially immobilized cells can then be used in bioreactors under controlled conditions that are favorable not only for optimum growth of organisms but also for the complete decolorization/mineralization of dye-contaminated wastewater.

Conclusion

The probable organism isolated that helped in decolorization of 0.005% may be of Gram-positive species, for 0.01% concentration may be *Pantoea agglomerans* and for 1% may be *Escherichia sp*.

The organisms isolated were subjected to Dye Decolorization Assay to evaluate their dye decolorizing ability. The Percentage dye decolorization activity was found to be 94%, 28%, and 0.07% for concentrations 0.005%, 0.01%, and 1% of Coomassie brilliant blue-G respectively. As this is an issue of environmental pollution, the probable isolates can play an important role in the prevention of pollution. It can be concluded that dye decolorization can be a solution for the treatment of wastewater and textile effluents which are alarming for the environment. In future studies, the characterization, optimization, and molecular investigation of the azo dye degradation by the probable isolate can also give vital information in this field to solve the arising problem. We can also develop molecular biology techniques and commercially can produce such enzymes to protect the environment from pollution.

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