Isolation of Congo red dye degrading bacteria from effluent, screening & evaluation of their dye decolorization activity.

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Rapid industrialization has led to accumulation of various toxic elements that harm the environment and ultimately affect the 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 deal 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 environment conditions. Media consisting of Congo red at different concentrations ranging from 0.005%, 0.01% and 1 % were used in order to isolate dye degrading organisms from effluent sample. Colony characteristics of organisms obtained from medium containing Congo red were studied and biochemical tests i.e. IMViC, TSI were performed for identification. Dye decolorization assay was performed colorimetrically at 465 nm for concentrations of Congo red ranging from 0.005%, 0.01% and 1% every 24 hours. The percentage of dye decolorization activity was found to be 97%, 61% and 0.05% for Congo red for concentrations ranging from 0.005%, 0.01% and 1% respectively. The probable isolated organism from Congo red i.e. *Pantoea agglomerans* was found to possess the ability to decolorize Congo red at lower concentration. The probable isolates obtained needs to be further investigated regarding various factors such as media composition affecting dye degradation & mechanism of dye degrading activity.

Keywords: - Azo dyes, Bioremediation, Dye decolorization assay, *Pantoea agglomerans*, Congo red.

INTRODUCTION

Rapid industrialization has led to accumulation of various toxic elements that harms the environment and ultimately affecting the human lives. Industrial processing often leads to effluents which consists 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. It necessary that every industry must have its own waste water treatment plant. But most of the industries lack this and thus waste water is directly let out into water bodies without any treatment. [1]

Textile dye effluents generally consist of toxic dyes and heavy metals. In ancient age natural sources were used for dying clothes. But the extraction process was a bit difficult and also expensive. Hence there was a need of 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 then in use. [1] According to annual report of the Union Ministry of Environment and Forest, some 4.4 million tones 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 the 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 consists 0.6–0.8 g^{L-1}. [5] There are various methods that can be used for removal of the dyes from the wastewater. [6] These methods include physical, chemical and biological methods that help in decolorization. Physical method generally includes coagulation-flocculation but is less efficient in dye removal process from wastewater. Also, it tends to produce high amounts of sludge [7]

Adsorption method includes using an adsorbent that can adsorb the dye molecules on the basis of affinity in the wastewater.[8] Activated carbon acts as the best adsorbent but is expensive.[9] Other low cost substitute can be used such as wheat straws, maize stalks, peat, clay etc but regeneration and disposal is tedious 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 has other limitation such as high cost of membranes, membrane fouling etc.[12] Chemical methods generally uses oxidizing agents such as ozone (o_3), hydrogen peroxide (H_2O_2) and permanganate (MnO_4) but has limitations such as high cost, low COD removal capacity etc.[6] Thus, physical and chemical methods are either costly or are inefficient in complete dye removal or tends 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 waste water and has certain advantages over physical and chemical methods such as a) eco friendly b) low-cost c) less sludge production d) non toxic 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*) have the ability to degrade azo dyes under anaerobic conditions.[1] Bacteria are widely used as many of them posses the ability to degrade the azo dyes and eliminate them through wastewater and 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 of the industries rely on enzymatic technology to treat the dye contaminated wastewater. Basically, there are two kinds of enzymes that lead to the decolorization viz. reductive enzymes and oxidative enzymes. [17]

Reductive enzymes

Flavin dependent and non flavin 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 first step azo dye gets converted to hydrazine and in second step hydrazine gets converted to two constitutive amines. The flavin dependent azoreductases are considered to be polymeric in nature and also provides thermal stability to 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 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 plays major role in dye decolorization activity.Peroxidase substrates are been degraded by peroxidase enzymes. Lignin peroxidase and veratyl alcohol oxidase in combinations increases the efficiency of dye degradation activity of azo and anthroquinone dyes. Also mono-rhamnolipid like molecules increased the activity of lignin peroxidase and vertayl alcohol

which may be as these molecules may provides orotection from inactivation of enzyme by hydrogen peroxide or any other factor. Telke et al. (2009) reported a novel enzyme, a laccase-like phenol oxidase that has the ability to 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 helps accelerating the reaction process. These laccase mediators are considered to be good substrates as they have the ability to be stable even in 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 Department of Biotechnology, Smt. Chandibai Himathmal Mansukhani College, Ulhasnagar-3, District Thane, Maharashtra, India

. 1. Sample Collection [28]

Textile effluents are directly released into Ulhas River. Thus, 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 its physico-chemical 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% Congo red, for 1 week. A loopful from the same, was streaked on sterile nutrient agar plates containing 1% Congo red. Similarly, a loopful of effluent sample was directly streaked on sterile nutrient agar plates containing 0.005% and 0.01% of Congo red. All the plates were incubated at room temperature/24hours.

b) Secondary Screening of dye decolorizing strains [28]

The isolated colonies obtained from above plates were again streaked on sterile nutrient agar plates containing 0.005%, 0.01% and 1% Congo red. Plates were incubated at room temperature. The

isolated organism were streaked on sterile nutrient agar slants containing 0.005%, 0.01% and 1% Congo red 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 Congo red was incubated at room temperature for 10 days. 5ml was withdrawn every 24 hours and centrifuged at 2500 rpm and was subjected to colorimeter to read optical density at 465nm.

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 Ulhas River at Ulhasnagar. Thus, only one effluent sample from Ulhas River, Ulhasnagar East was used.

Isolation of organisms from effluent sample on Nutrient agar with respective dye concentration as follows:-

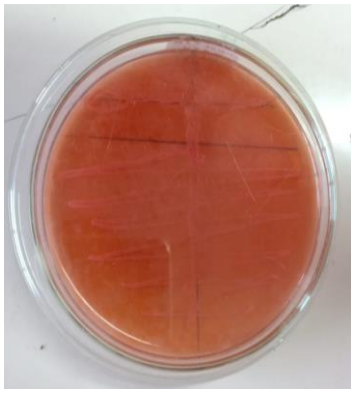

Figure no. 01

Growth obtained on sterile nutrient agar plate containing 0.005% Congo red

Figure no. 02

Growth obtained on sterile nutrient agar plate containing 0.01% Congo red

Figure no. 03

Growth obtained on sterile nutrient agar plate containing 1% Congo red

Biochemical tests – IMViC and TSI test

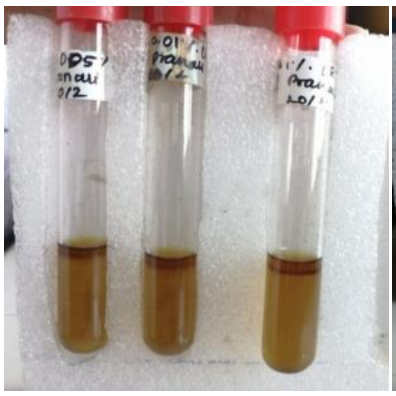

Figure no. 04

Indole test for identification of isolates obtained on plate with 0.005%, 0.01%, 1% Congo red respectively.

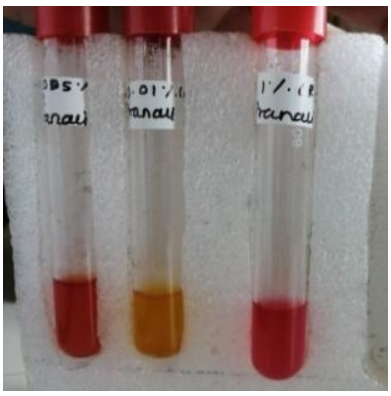

Figure no. 05

Methyl red test for identification of isolates obtained on plate with 0.005%, 0.01%, 1% Congo red respectively.

Figure no. 06

VP test for identification of isolates obtained on plate with0.005%, 0.01%, 1% Congo red respectively.

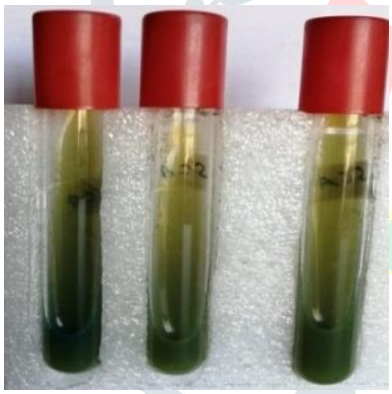

Figure no. 07

Citrate utilization test for identification of isolates obtained on plate with 0.005%, 0.01%, 1% Congo red respectively.

Figure no. 08

TSI test for identification of isolates obtained on plate with 0.005%, 0.01%, 1% Congo red respectively.

Table no. 1- Prediction of probable organism isolated on sterile nutrient agar plates containing 0.005%, 0.01% and 1% Congo red concentration on the basis of IMViC and TSI test.

Sr. No	Media with respective concentrations of dyes	Probable organism isolated
1.	Nutrient Agar + 0.005% Congo Red	*Pantoea agglomerans*
2.	Nutrient Agar + 0.01% Congo Red	*Pantoea agglomerans*
3.	Nutrient Agar + 1% Congo Red	*Pantoea agglomerans*

Dye decolorization assay

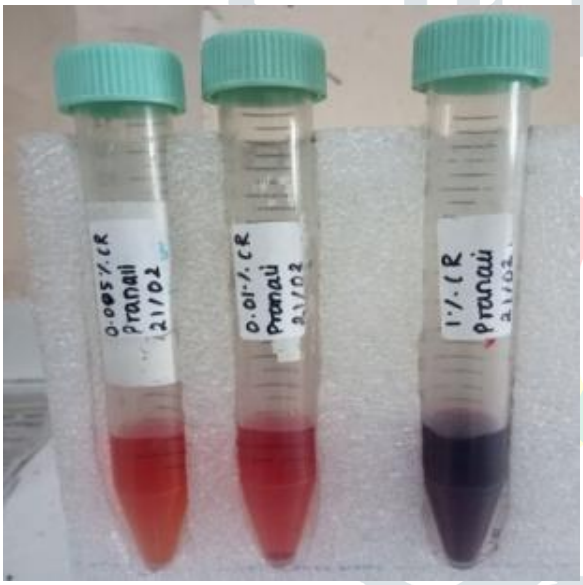

Figure no. 09

Day 0 - 0.005%, 0.01% and 1% Congo red respectively.

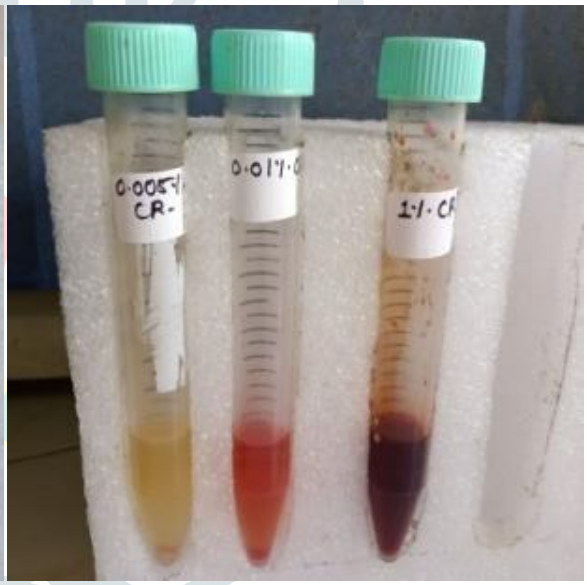

Figure no. 10

Day 10 - 0.005%, 0.01% and 1% Congo red respectively.

Table no. 2 - Dye decolorization assay results

Sr.no	No of Days	O.D 465 nm		
		0.005% Congo Red	0.01% Congo Red	1% Congo red
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	>1.0	>1.0	>1.0
5.	Day4	>1.0	>1.0	>1.0
6.	Day5	0.52	>1.0	>1.0
7.	Day6	0.50	>1.0	>1.0
8.	Day7	0.49	>1.0	>1.0
9.	Day8	0.11	>1.0	>1.0
10.	Day9	0.09	>1.0	>1.0
11.	Day10	0.06	>1.0	>1.0
Percentage Of Dye Decolorization		97%	61%	0.05%

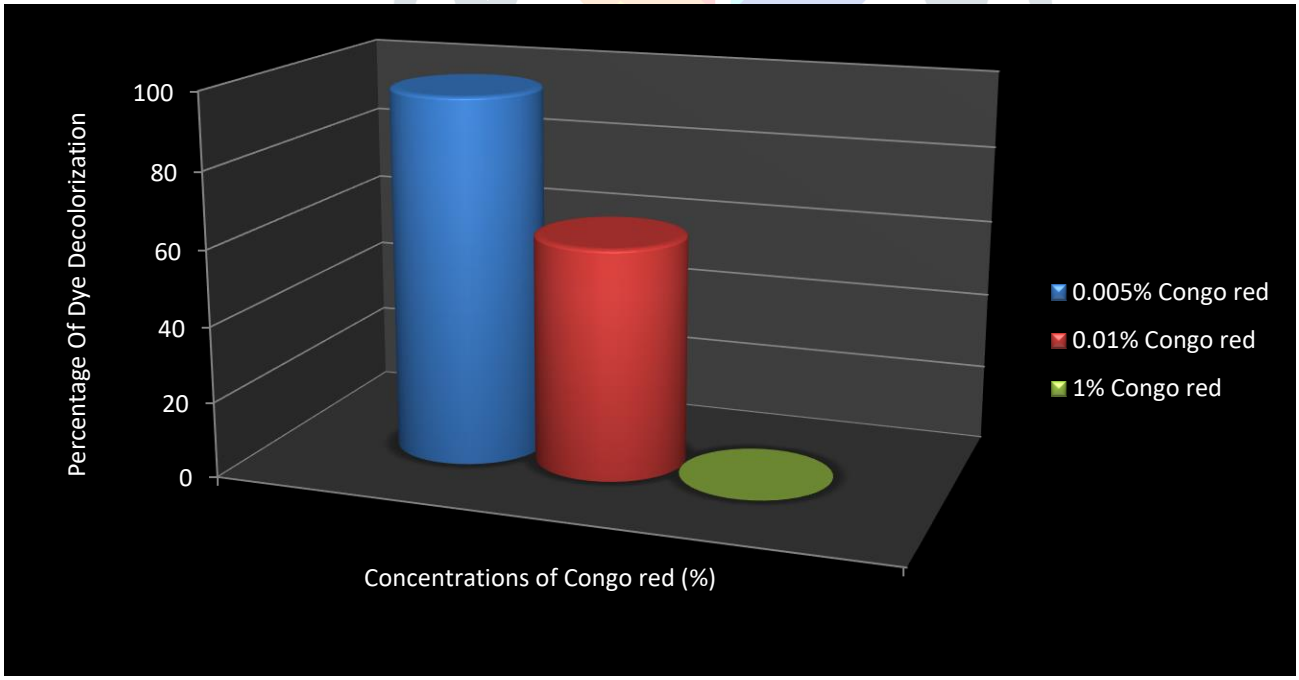

Chart no. 1- Graphical Representation of Dye Decolorization Assay for 0.005%, 0.01% and 1% concentration of Congo red (465nm)

DISCUSSION

Dye contaminated wastewaters are not only harmful for aquatic life and environment but are also harmful for 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%, 0.01% and 1% Congo red was found to be *Pantoea agglomerans*.

The percentage of dye decolorization activity was found to be 97%, 61% and 0.05% for Congo red for concentrations ranging from 0.005%, 0.01% and 1% respectively. Degradation of Congo red 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 decrease in color intensity observed. A control flask was kept, which contained only nutrient broth & respective concentration of dye. It was observed that the dye intensity & O.D was constant throughout the incubation period that is Day 0 to day 10. This indicates that the dye is not getting degraded spontaneously but it is getting degraded due to microorganisms in the medium.

Study conducted by Alyssa M. Walterson and John Stavrinides reveals that 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 biodegradation and bioremediation of toxic products released from industries into the environment. [18] 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 enzymes. The enzymatic activity was found to be NADH dependent and the enzyme was monomeric flavin-free azoreductase. It helped in 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 in order to decolorize the azo dye i.e. Methyl red at various concentrations. The fluidized bed bioreactor was prepared in 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 rotary shaker at 100 rpm (25^{oC}). For immobilization of cells calcium alginate, polyacrylamide, cooper beech, and 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]

A study was conducted by Niranjan Prakashrao Patil, Jumma Shaikh, B.P. Kapadnis and V.B. Gaikwad to evaluate the decolorization of dyes and its mixture by *Providencia sp.* Dyes used were DM10 and Malachite Green. The optimum condition for decolorization of DM10 was found to be static/anoxic incubation, pH 7, 35°C temperature, 1.5% (w/v) NaCl and 150 ppm DM10 concentration. Laccase, lignin peroxidases, tyrosinases (oxidative) and azo reductase (reductive) enzyme activities were responsible for decolorization of DM10 dye.[21]

In a study conducted by Harshad Lade, Sanjay Govindwar, and Diby Paul on Low-Cost Biodegradation and Detoxification of Textile Azo Dye C.I. Reactive Blue 172 by *Providencia rettgeri* Strain HSL1 reveals using agricultural waste wheat bran (WB) which can be used as a cheapest source of growth medium for degradation of textile azo dye Reactive Blue 172 (RB 172) . 50 mg L^{-1} of dye RB 172 was decolorized by the bacterium within 20 h at 30 ± 0.2°C under microaerophilic incubation conditions. It was found that azoreductase (159%) and NADH-DCIP reductase (88%) produced was responsible for complete decolorization of the dye. HPLC, FTIR, and GC-MS analysis of the decolorized end product confirmed the degradation of dye. Hence, wheat bran can be used as an low cost growth medium for enrichment for decolorization of dyes. [22]

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 the Glucose and ammonium sulphate 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 needs 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 needs to be studied, identified and evaluation of it is required. Also, various conditions that lead to higher biomass concentration also need to be evaluated. The products that are produced after 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.

CONCLUSIONS

Isolation of organisms was done on sterile nutrient agar plates containing 0.005%, 0.01% and 1% Congo red. The probable organism isolated that helped in decolorization of 0.005%, 0.01% and 1% Congo red was found to be *Pantoea agglomerans*. The organisms isolated were subjected to Dye Decolorization Assay in order to evaluate their dye decolorizing ability. The Percentage dye decolorization activity of probable organism *Pantoea agglomerans* was found to be 97%, 61% and 0.05% for concentrations 0.005%, 0.01% and 1% of Congo red 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 the dye decolorization can be a solution for the treatment of waste water and textile effluents which are alarming for the environment. At 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 technique and commercially can produce such enzyme to protect environment from the pollution.

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