Journal of Engineering Sciences
Vol 11, Issue 6,June/2020
ISSN NO:0377-9254

MULTIDRUG RESISTANCE IN DYE DECOLORIZING MICROBES

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Abstract— The triphenylmethane dyes are highly soluble in water and has long been used extensively for dyeing silk, wool, jute, leather, ceramics, and cotton. Although used widely, most of these dyes are toxic, carcinogenic and hardly biodegradable because of their complex aromatic structure. In this study, we have isolated two microorganisms from dye contaminated sites, which showed good capacity of decolorization of triphenylmethane dyes like Malachite Green, Crystal Violet, Basic Fuchsin from solution. They were equally potent in decreasing COD and TDS of dye solutions thus possess the capacity of bioremediation of toxic triphenylmethane dyes. These two qualities have made the organisms excellent possible choices to be used industrially in the future. However, as they are obtained from natural sources, we analysed them for their sensitivity towards a number of antimicrobial drugs and both of them were found to be resistant against multiple drugs. Although antibiotics are hardly used by the dying industries, presence of multidrug resistant organisms are not only surprising but also pose potential threat of spreading antibiotic resistance both naturally and if used industrially for dye decolorization purpose.

Keywords— Dye, Decolorization, COD, Bacteria, Multidrug Resistance

1. INTRODUCTION

Among the many contaminants present in wastewater, dyes and colors comprise of one of the biggest group compared to others such as acids, bases, toxic organic and inorganic dissolved solids. Common dyes which are used industrially usually are synthetic in nature and they have complex aromatic molecular structure which make them more stable and very hard to biodegrade[1]. It has been estimated that approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually worldwide [2]. These dyes have also been found in soil and river sediments decolorization of dye waste waters [4,5,6] It is turning into a promising and green alternative to replace or supplement present treatment processes.

Although a good number of successful research works have been done on isolation of dye degrading microbes, not much research work has been progressed to determine spread of antibiotic resistance through these organisms while inhabiting their natural habitat or while used industrially. Antibiotics are special group of therapeutic agents which are used for the treatment of diseases caused by different microorganisms. However, the indiscriminate and intensive use of antibiotics resulted in accumulation of those antibiotic in the environment in the un-metabolized form. This in turn, leads to large residues of antibiotics in the recipient waters and emergence of antibiotic resistant microbial strains [7]. Antibiotic resistance is not only confined to bacteria in a medical surroundings but is also found in environment. Several studies have showed the spread of antibiotic resistance genes, as well as, multidrug resistant (MDR) bacteria from clinical and communal effluents to the various receiving water bodies [8,9]. Those as a consequence of improper chemical disposal, leading to reduction of sunlight penetration, which in turn decreases both photosynthetic activity and dissolution of oxygen concentration[3].

Bacteria that grow naturally in dye contaminated sites inherit innate capacity of tolerating high concentrations of dyes. Therefore, indigenous bacterial isolates from dye contaminated place can be a welcoming tool to decolorize as well as remove these recalcitrant dyes from the environment. In recent years, there has been an intensive research on microbial

antibiotic resistant bacteria from the environment can become a major threat to humans, since they can act as a pool for the spread of antibiotic resistance[10]. In most developing countries, where effluents are discharged from the sewer systems directly into rivers and lakes, these antibiotic resistant bacteria can be a major cause of concern as they can be disseminated and can potentially transfer their resistance through horizontal gene transfer [11].

Microorganisms, which are helpful from the point of industrial usage, can become an unknown nuisance and spread antibiotic resistance to all the flora residing in dye contaminated wastewater as well as the sewage water when they get mixed to sewage during their release.

In this study, antibiotic sensitivity profile has been determined for two bacterial species, isolated from dye contaminated wastewater, for the treatment of triphenylmethane dyes. These organisms were found to be - *Enterobacter asburiae* (*E. asburiae*) XJUHX-4TM and *Klebsiella pneumoniae* (*K. pneumoniae*) -1TM by sequencing their 16S rRNA [12]. Both of these organisms were found to be highly competent in

decolorizing the Triphenylmethane dyes (TPM dyes) like Malachite Green, Crystal Violet and Basic Fuchsin , which are considered potent carcinogens and threat to the environment [13,14]. These two were found to be equally efficient in reducing the COD and TDS of dye containing solutions. These two prospective decolorizer of triphenylmethane (TPM) dyes were screened for their sensitivity and resistance against a number of antibiotics. When these organisms are present in natural environment they have a spontaneous capability to decolorize the colored effluent dumped in that area, and under these circumstances, if they have antibiotic resistance present in them, they can unknowingly spread the resistance easily, making other organisms resistant as well. Hence, if they are used for industrial dye removal purpose, it is necessary to determine their drug sensitivity profile to be assured of the possible threat when used in large scale.

2. MATERIALS AND ETHODS

2.1. ISOLATION, ACCLIMATIZATION AND IDENTIFICATION OF THE MICROORGANISM

Dye contaminated wastewater was collected from a small scale textile industry situated at Habra, West Bengal and the sample was serially diluted and spread on Mineral salt agar medium plates [Composition (g/L): Sucrose – 15, KCl - 0.5, $NaNO_3$ - 2 , $MgSO_4$, $7H_20$ - 0.5, $FeSO_4$, $7H_2O$ - 0.01, agar - 20 and TPM dyes of varying amounts separately and in a mixed solution (prepared by dissolving Malachite Green, Crystal Violet and Basic Fuchsin in equal proportion ,range 0.01-0.5 g/L),pH = 7 ± 0.2] . The TPM dyes were given in very little concentrations initially and the concentrations were increased gradually. The bacterial colonies capable of growing in this above mentioned media were then acclimatized accordingly to higher concentrations of dyes. Almost 20 colonies were randomly selected for the purpose of acclimatization depending on various colony characteristics. Among them 2 colonies showed consistent decolorizing capacity of TPM dyes up to 500 mg/L. Identification of the selected strains were carried out according to 16S rRNA analyses. The most potent organisms were found to be *Enterobacter asburiae*-XJUHX-4TM and *Klebsiella pneumonia*-1TM [12]. These two bacterial species were then utilized for studying decolorization of TPM dyes in Mineral Salt broth medium (Composition same as mineral salt agar medium without agar).

2.2. BATCH DECOLORIZATION PROCESS

The experiments were conducted in 250 ml Erlenmeyer flask containing 100 ml of mineral salt medium containing dyes, which was inoculated with 1 ml log phase culture of *E.asburiae*-XJUHX-4TM and *K .pneumoniae* -1TM cells containing 0.9×10^9 cells separately. The experiment was conducted at its experimentally determined optimum conditions [12]. The decolorization studies were carried out to study the decolorization of Malachite Green, Crystal Violet, Basic Fuchsin and mixed dye solution. The decolorization was carried out at pH 8, temperature of 35ºC and maintained under shaking condition at 130 rpm for 24 hrs. After complete decolorization, the dye removal was determined by % decolorization of the dye.

2.3. Measurement of dye decolorization

After the growth and decolorization by the microorganisms, the mineral salt medium containing dyes were centrifuged at 8000 rpm for 10 min. Clear supernatant was used for determination of dye removal in terms of % decolorization spectrophotometrically (Elico SL 210). Malachite Green , Crystal violet and Basic Fuchsin (procured from SRL) – the pure dye solutions separately and also in a mixture were scanned from 300-700 nm, where from maximum absorption (λ_{max}) was obtained at 617 nm, 590 nm, 540 nm and 585 nm respectively for Malachite Green, Crystal Violet, Basic Fuchsin and dye mix. The medium supernatant was used to determine decolorization efficiency both before and after bacterial treatment separately at λ_{max} characteristic for each dye. The % decolorization was calculated using the following expression:

% decolorization = [(initial absorbance-observed absorbance) /initial absorbance] ×100

2.4. DETERMINATION OF TDS AND COD

As various dye industries use various types of dyes for different dyeing purposes, it is difficult to measure pollution load visibly and with the help of spectrophotometer alone. To be more specific, the total dissolved solids (TDS) and Chemical Oxygen Demand (COD) are usually considered as appropriate parameters for determining pollution load imposed by the dyes and pigments to any wastewater.

In this case, the mixture of dye solutions was used for determination of TDS and COD using the protocol prescribed by APHA 1998 [15].The mixed dye solution, before and after treatment with *E.asburiae* and *K. pneumoniae* were used to determine TDS and COD in order to assess the potentiality of the organisms to decrease the pollution load. In each case, the percentage decrease in TDS and COD were determined.

2.5. Determination of antibiotic resistance

The antibiotic sensitivity test for the bacterial isolates were carried out by the modified disc diffusion method of Bauer and Kirby using Muller Hinton medium against the following antibiotic discs: Cloxacillin (10 µg), Ampicillin (10µg), Penicillin (10µg), Methicillin (10 µg), Amikacin (30µg),

streptomycin (30µg) Nalidixic acid (30 µg), Tetracyclin (30µg), and Chloramphenicol (30µg) (Merck India) . The plates were first swabbed with the cultures and then antibiotic discs were applied, followed by 24 h incubation at 37 ° C. The zone of diameter of inhibition was measured in cms and depending on the available data of CLSI (Januay2011) [16], their sensitivity was determined. The results are average of triplicate sets ± standard deviation and each inhibition zone was measured from three orientations and the average was considered.

2.6. Determination of multiple antibiotic resistance (MAR) index

The Multiple Antibiotic Resistance (MAR) index for the two organisms were determined by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested [17, 18] – MAR index = Number of antibiotics isolate is resistant to / Total number of antibiotics tested.

3. Results and Discussion

3.1. Potency of dye removal by the two strains:

Both the bacterial strains were found to be quite efficient in decolorizing triphenylmethane dyes, separately as well as from mixture. However, *Enterobacter asburiae* -XJUHX-4TM was found to be a bit better as a decolorizer than *Klebsiella pneumoniae* 1TM. *Enterobacter* was found to decolorize dye mix upto a concentration of 500 mg/L, whereas, the latter one, *Klebsiella pneumoniae* , although showed potency of dye removal up to the same, but with decreased efficacy (Fig. 1 and 2). Both the organism showed better efficiency when used for single dye solutions. The decolorizing potency decreases as the dye concentrations increases in both the cases (Fig. 1 and 2). [19] suggested that the decrease in decolorization capacity might be due to the toxicity imposed by the dyes on organisms, which is an important consideration for its field application. Initial dye concentration is required to be considered in dye decolorization experiment as it is needed to overcome all mass transfer resistance of the dye between the aqueous and solid phases [20]. The efficiency of reduced decolorization could be explained by occupancy of the active dye binding sites on the microorganisms by dye molecules, as the concentration of dye mix increases.

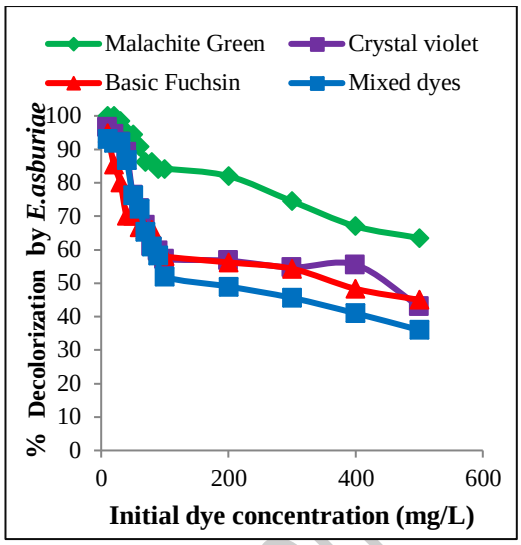

Fig 1. Effect of initial dye concentration on dye decolorization for each dye by *Enterobacter asburiae* -XJUHX-4TM

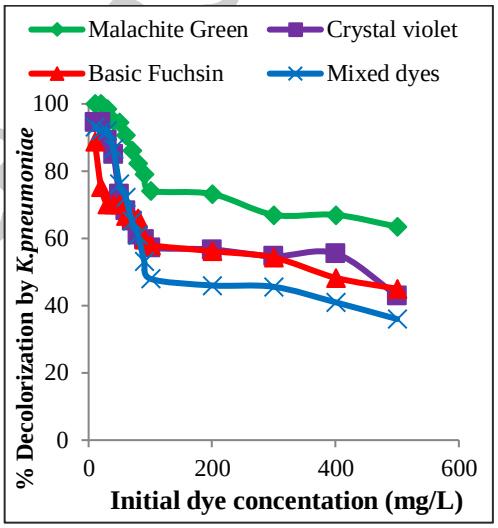

Fig. 2. Effect of initial dye concentration on dye decolorization for each dye by *Klebsiella pneumoniae* 1TM

3.2. Reduction in COD and TDS level

Along with a satisfactory results obtained in terms of decolorization, a good amount of decrease in COD and TDS were also observed for both the organisms. In case of both COD and TDS, an appreciable reduction was obtained at lower dye concentration, almost 50 percent decrease in both the parameters were obtained till 100mg/L (Fig, 3 and 4) dye concentrations.

As concentrations increases beyond 100 mg/L, no appreciable level of reduction of COD and TDS were noted. We found that as dye concentrations increase, decolorization efficiency decrease, which may directly impact the observed reduction in COD and

TDS level.

Overall, in terms of decolorization of TPM dyes and reduction in COD and TDS level, we see *Enterobacter asburiae* -XJUHX-4TM and *Klebsiella pneumoniae* 1TM were quite efficient, which indicate their potency to be used as a source of bioremediation of water contaminated with TPM dyes.

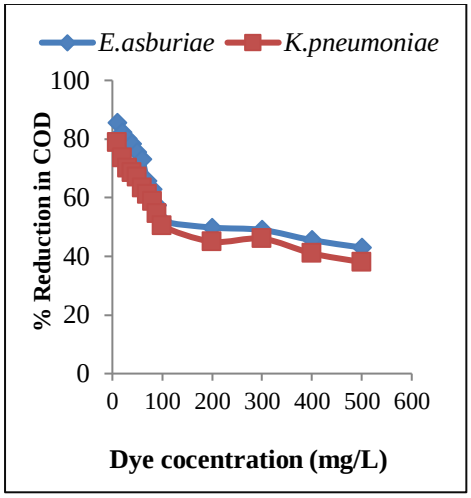

Fig. 3. % Reduction in COD of mixed dye by *Enterobacter asburiae* -XJUHX-4TM and *Klebsiella pneumoniae* 1TM

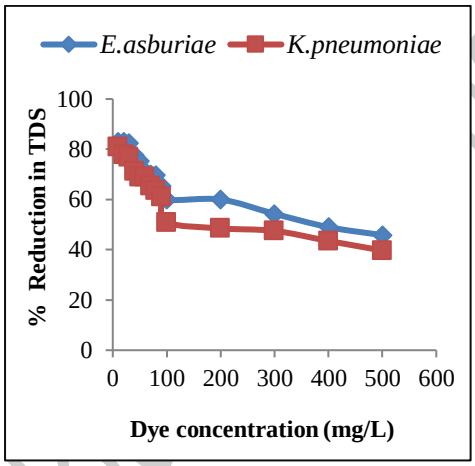

Fig 4. % Reduction in TDS of mixed dye by *Enterobacter asburiae* -XJUHX-4TM and *Klebsiella pneumoniae* 1TM

3.3. Antibiotic sensitivity pattern of the organisms:

These days the environment around is so polluted and toxic, that they can affect the natural microflora inhabiting there and can mutate the natural species in such a way that a simple microorganism can become a severe 'superbug'.

Although efficient in terms of industrial dye decolorization, the two organisms isolated from environmental samples, must be monitored in terms of their antibiotic sensitivity before being applied industrially. Hence antibiotic sensitivity assay was conducted for the two organisms. The antibiotic sensitivity profile of the two species according to CLSI is given in Table 1.

From the table, it has been noted that these two microorganisms do possess resistance against a number of antibiotics like ampicillin (amp), penicillin (pen), cloxacillin (clox), methicillin (met) – mostly beta-lactam antibiotics and also to nalidixic acid (NA) and sensitive towards amikacin (amk), tetracycline (tet) and streptomycin (str) and chloramphenicol - which is a matter of concern if applied in the field directly for dye decolorization purpose. It can not only spread antibiotic resistance to the microorganisms indigenous to the place, but can also very easily affect the people working with the organisms.

3.4. Determinations MAR:

In this study, we have used five different types of antimicrobial agents, viz., beta-lactam antibiotics (penicillin, ampicillin, methicillin and cloxacillin), aminoglycosides (amikacin, streptomycin), tetracycline, chloramphenicol and nalidixic acid. From Table 1, we can clearly see that *Enterobacter asburiae* is resistant to beta-lactams penicillin, cloxacillin,methicilin and nalidixic acid amng the 9 antibiotics used, whereas, *Klebsiella pnumoniae* is resistant to penicillin, cloxacillin, ampicillin and nalidixic acid among the nine. For both the bacteria, MAR index was found to be 4/9=0.67 (Table 2). MAR index higher than 0.2 has been said to be an indication of isolates originating from an environment where antibiotics were often used [17] . In bothe the cases the index is fairly high. It is quite surprising, that a small scale dye industry can also be affected by indiscriminate usage of antibiotics, because normally we know that dye industries have nothing to do with the antibiotics.

Table 1 – Antibiotic sensitivity profile of the two bacterial strains

Name of the organism	**[Zone of inhibition (cms) ± SD]**				
	Amp (10 mcg)	**Clox (10 mcg)**	**Pen (10 mcg)**	**Met (10 mcg)**	**Amk (30 mcg)**
E. asburiae	I (1.4±0.2)	R (1.3±0.11)	R (1.2±0.21)	R (0.9±0.03)	S (2.2±0.08)
K. pneumoniae	R (1.2± 0.09)	R (1.3±0.13)	R (1.3±0 .21)	S (1.1± 0.06)	S (2.1 ± 0.17)
Name of the Organism	**Chl (30 mcg)**	**NA (30 mcg)**	**Tet (30 mcg)**	**Str (10 mcg)**	**S.D. water (Control)**
E. asburiae	S (1.8 ±0.13)	R (1.2±0.21)	S (1.7±0.15)	S (1.9±0.22)	R (No inhibition)
K. pneumoniae	S (1.7±0.1)	R (1.3±0.15)	S (1.8± 0.11)	S (2.1±0.15)	R (No inhibition)

[R= Resistant ; S= Sensitive; I = Intermediate]

Table 2 – Determination of MAR index of the two bacterial strains

Name of organisms	**MAR index**
E. asburiae XJUHX-4TM	4/9 = 0.67
K. pneumoniae 1TM	4/9 = 0.67

Conclusion

From the studies carried out it is clearly noted that the two bacterial species, *Enterobacter asburiae*-XJUHX-4TM and *Klebsiella pnumoniae*-1TM can be considered as potent decolorizer of TPM dyes. They decolorize the dye solutions separately and also in a mixed solution quite efficiently till a concentration of 500 mg/L. Additionally, both of them were found to be proficient in terms of reduction of COD and TDS of mixed dye solutions. Therefore, from industrial point of view it can be considered that these two organisms can be used efficiently for the treatment of industrial effluent containing TPM dyes with a decrease in environmental pollution load. However, before applying these microbes indiscriminately for the above mentioned purpose, it has to be noted that although they are competent from the economic point of view, in effective treatment of wastewater using microbes due to their inherent quality of producing

minimal amount of sludge as byproduct, yet these organisms can be a potential threat for spreading antibiotic resistance. Although these two species have been isolated from dye contaminated wastewater, where use of antibiotic is practically very much limited, still the high MAR value of the organisms indicate indiscriminate use of antibiotics and their mixing with environmental discharge, that results in transformation of natural ,innocent microbes that could be used for industrial dye removal purpose into posing risks of being superbugs and becoming a cause of global antibiotic resistance.

Acknowledgement

The author is highly grateful to Scottish Church College authority and all staff members of Microbiology Department of Scottish Church College for their support and University Grant Commission for providing financial assistance to conduct the Minor Research Project under F.PSW-123/15-16 (ERO).

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