BIODEGRADATION OF MALACHITE GREEN DYE BY MICROBES ISOLATED FROM TEXTILE INDUSTRY EFFLUENTS.

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ABSTRACT

We have isolated a bacterial strain Pseudomonas, which could degrade malachite green upto-80 µM. The activity of triphenylmethane reductase enzyme responsible for decolorizing triphenylmethane dyes was estimated. The enzyme involved in the degradation was estimated. A gene encoding the enzyme was amplified based on its N-terminal and internal amino acid sequence. PCR products were sub cloned to T-DNA Vector and the gene coding for triphenylmethane reductase was amplified and submitted for sequencing.

Key Words: Malachite green, Bacterial strain, Degradation, Encoding and T-DNA Vector.

INTRODUCTION

Dyes are coloring pigments that impart color to the substrate when they are in solution form. Technically dyes are distinguished from the intermediates based on the presence of auxochrome, the group that allows the basic unit to attach and impart color to the substrate. Dyes are derived synthetically from raw materials like hydrocarbons, benzene, toluene, naphthalene and anthracene using coal tar obtained from distillation of coal (Mittal et al 2005).

Both organic and inorganic materials are needed to make dyes and intermediates. The raw material sequence for making dyes is petroleum --hydrocarbons ---intermediates --dyes. Dyes are retained in substrates by physical absorption, metal complex formation or by the formulation of covalent chemical bonds and they obtain their color due to electronic transitions between various molecular orbital where intensity of the color is determined by the probability of transitions (Mojsov et al.,2016). Dye is a substance (generally an organic compound), which is used for imparting permanent color to textiles - silk, wool and other substances.

Triphenylmethane dyes have been found in soil and river sediments as a consequence of improper chemical waste disposal e.g. malachite green has been found in sediments from the Buffalo river a tributary of Lake Erie was shown to be carcinogenic (U.S. Environmental

Protection Agency, Genetox' Clastogen, 1986) and crystal violet has been shown to be a potent clastogen. This class of chemicals is reported to be responsible for the promotion of tumor growth in some species of fish. The conventional wastewater treatment systems are unable to remove recalcitrant dyes from the effluents.

Some of the triphenylmethane dyes are used in medicine as biological stains, in paper and leather industry; for coloring plastics, in gasoline, paper and leather industries are the major consumer of azo and triphenylmethane dyes (Mondal et al 2017). Food and cosmetic industries also use different types of dyes. 10,000 dyes and pigments are produced annually worldwide amounting to 7x105 tones which are hazardous and pose serious environmental problems. It is estimated that 10-15% of the dye is lost in the effluent during the dying process.The recent high profile of color pollution is mainly the result of increasing public awareness and expectations of the environment; coinciding with rising levels of color discharges.

One of the more pressing environmental problems that have been facing the textile industry is the removal of the color from dye bath effluent prior to discharge to local sewerage treatment facilities or adjoining watercourses. Considerable efforts have been made on developing suitable treatment systems for these effluents. Only biotechnological solutions can offer complete destruction of the dye stuff with a co-reduction in the biological oxygen demand (BOD) and chemical oxygen demand (COD).

Wastewaters originating from reactive dye processes have created a particular problem because the dyes can exhibit low levels of fixation with the fiber. The brightly colored unfixed dyes are highly water-soluble and are not removed by conventional treatment systems. This is particularly noticeable as the human eye can detect reactive dyes at a concentration as low as 0.005 mg/l in clear waters. Discoloration of textile dye effluent does not occur when treated aerobically by municipal sewerage systems.

TOXICITY OF MALACHITE GREEN:

Besides the problem of color there is concern that some azo dyes either are toxic or carcinogenic compounds. There are no universally useful methods available for the treatment of dye wastes. Probably because of the complex and very varied chemical structures of these compounds and few of the currently used biological treatment methods can be successfully employed.

Churchman and Herz (1913) reported first case of toxicity of Gentian violet (an impure form of Crystal violet) and its fate in animal body. They did a series of seventy-five experiments on dogs and rabbits to observe the bactericidal property observed by Gentian violet.For the intravenous infection of Gentian violet rabbits were used. The dye in varying concentrations was ejected into the ear vein. The cornea, conjunctiva, mucous membranes of both mouth and lips immediately became blue.

It is evident that Gentian violet injected intravenously into rabbits disappears from the blood in a short time and there is no similar loss selective bactericidal power. When the dye is simply allowed to remain in contact with the blood in vitro. On painting the towns of dog with strong solution of the dye frozen section showed that penetration occurred through the thickness of mucosa down to muscularis.

When malachite green is used in aquatic animals, it will be metabolized to leuco-malachite green. The non-polar LMG has been found to retain in catfish muscle for a longer period of them, 10 days for LMG compared to 2.8 in MG. It has been determined that the half-lives of the retention of malachite green and leuco-malachite green catfish muscle is 2.8 days.

The study of the toxicity of malachite green in fish has been hard as it is heavily influenced by the water hardness, pH, temperature and amount of dissolved oxygen in water. Detailed studies have indicated that the toxicity of the chemical increases as the temperature increases or pH decreases. The effects of malachite green on fish eggs have also been tested and it has been shown that a twofold increase in the concentration of malachite green could lead up to 20 times the mortality rate in rainbow trout eggs. This shows that it may be extremely toxic for some species of fish and especially for fish eggs. Other effects such as carcinogenesis, mutagenesis, and reduced fertility have been reported to occur in rainbow trout.

Nowadays Biological treatment methods are getting more attention since it is cheap and offer best alternative with proper analysis and environmental control. Almost all wastewaters can be treated by the use of a number of naturally occurring microorganisms such as bacteria and fungi. The color removal concentration in the textile industries varies between 1100-1300 mg/L. During coloration more than 15% of the dyes is wasted which enters the effluents.These effluents a source of environmental contamination characterized by high levels of chemical and biochemical (COD, BOD) oxygen demand, suspended matter solids, toxic and hazardous materials. The BOD of from dye waters typically varies between 200- 3000/ LSS and a pH of 4 – 12.

Primary stages are mainly physical and include screening, sedimentation, floatation and flocculation. The objective is removal of debris, undissolved chemicals and particulate matter. In the Secondary stage the organic load is reduced. Tertiary method involves adsorption, ion exchange chemical exudation, reverse osmosis etc.

BIOLOGICAL SYSTEMS:

87 dyestuffs were tested in short-term aerobic biodegradation tests to investigate whether some dyes might be susceptible to aerobic biodegradation and, if so, to what extent this occurs? For this work the dyestuff chosen were typical commercial products and bacteria inoculated were from effluent treatment plant. As in the static test, the criteria for biodegradation were both decolorization at the absorption maximum and dissolved organic carbon elimination. Their

results confirmed that dyestuffs are most unlikely to show any significant biodegradation in such tests.

DECOLORIZATION BY BACTERIA:

Although several Tri phenyl methane dye decolorizing bacteria have been isolated, there are few reports of specific enzymes that decolorize these dyes. The decolorization of malachite green and crystal violet by intestinal microflora and several anaerobic bacteria proceeds through enzymatic reduction to their respective leuco derivatives

However, the enzymes involved in this reduction have not yet been isolated or characterized in their purified forms. Their amino acid sequences and other biophysical parameters remain unknown. In the present study we tried to isolate the bacterial strains which had the capability to decolorize malachite green. We also estimated the enzyme that degrades the Triphenyl methane dye. We have also made an attempt at isolating and cloning the TMR gene in E.coli.

MATERIALS AND METHODS:

Source: Soil is collected from the textile industries in and around Hyderabad. This soil is then serially diluted and spread over nutrient agar plates for isolation of bacterial colonies.

Preparation of Nutrient Agar: Peptone 1gm, Yeast extract 1gm, NaCl 0.5gms and Agar agar 2gms.

The soil sample is again serially diluted and spread over a media (nutrient agar) along with a dye (malachite green 100mg/lt), and this is again repeated using another dye (crystal violet 50mg/lt)

ISOLATION OF BACTERIA: The colonies which have shown clear zone of clearance were picked and made pure culture using nutrient agar.

GRAM STAINING:

To identify the gram nature of the isolated organism, gram staining is done by Crystal violet, Grams iodine, Acetone and Saffranin.

Take a clean glass slide and place a drop of water over it. Now, sterilize the inoculating loop and pick a part of colony and spread over the water drop, make thin smears of it. Let the smears air dry and then heat fix. Stain the smear with crystal violet for 1-2 minutes, then wash it with distilled water, now cover the smear with grams iodine for about 1 min and wash the slide with distilled water, then add acetone (alcohol) drop by drop for about 30secs and immediately wash with distilled water. Lastly add saffranin to the smear for 2 minutes and wash with distilled

water. Blot dry the slide using a blotting paper. Let the slide dry completely and then observe the fluid under 10x, then observe morphology under 45x and finally report the gram morphology and arrangement under oil immersion lens (100x).

DISCOLORATION ASSAY: 100 ml nutrient broth was prepared and shared into four conical flasks (25 ml each.) with concentrations of malachite green (20µl, 40 µl, 60 µl, 80 µl) was added to each flask (25 ml). Inoculate a loopful of culture from the plates and incubate the flasks. Observe the OD values after 24 hrs of incubation at 600 nm.

IMVIC TESTS: To identify the three isolated organisms IMViC (Indole production, methyl red reduction, Voges Proskauer and citrate utilization) tests are done for each of the culture.

OXIDASE TEST: Pick up a 24hr old culture and smear the culture on Watt Mann filter paper and then impregnate with 2-3 drops of 1%oxidase reagent reduced with sodium thionate. Wait for 10 secs. If purple colour appears within 10 secs, it is a positive test for oxidase. But if the colour produces between 10-60 secs, it is delayed positive reaction while absence of colour is a negative reaction.

ENZYME ASSAY: The dyes taken are triphenylmethane dyes and the organism is able to reduce the dye, therefore triphenylmethane reductase enzyme assay is done. The standard assay system for TMR comprises 20mM sodium phosphate buffer (pH 7), 20µM dye (either malachite green or crystal violet), 0.1mM NADH and a suitable amount of culture broth in a total volume of about 1ml. this is incubated at room temperature for about 2 mins and then OD is observed at 600nm.

The enzyme activity is calculated as follows:

In general terms then enzyme activity (µmol /min/ml) = ΔA x 1000/ extinction coefficient of malachite green x volume in cuvette x 1.0/volume used for assay. The final activity obtained by multiplying by any dilution factor for micromoles per min per ml & dividing by the protein concentration in mg in per ml for micromoles per min per mg.

ISOLATION OF DNA:

Take 1.5ml of 24hrs old broth culture into tubes and spin at 1200rpm for 2mins and resuspend them in 100µl of distilled water. Then add 100µl of lysis buffer and mix immediately. Keep it in boiling water for 2mins and transfer the contents into a glass tube. Add equal volume of phenol and equal volumes of chloroform and isoamylalcohol in the ratio 24:1.Gently shake the contents of the tube with hand at room temperature for 20mins.

Now spin the tubes at 4000rpm for 30mins. The contents of the tubes separate into 3 layers- 1. Aqueous layer, 2. Cell debris layer and 3. Organic layer. Remove the aqueous layer into a fresh tube or flask and add 2 volumes of 95% ethanol and aspirate it with a Pasteur pipette. To the precipitate add 50µl of TBE buffer and read the observation in a uv spectrophotometer.

ESTIMATION OF DNA BY DPA (DIPHENYL AMINE) METHOD:

A liquid sample of standard DNA whose concentration is to be estimated is taken. The volume is made to 1ml by adding distilled water. 4ml of DPA is added to all the tubes. The contents of the tube are mixed thoroughly and heated for 10 mins in a water bath. The solution in the tubes turns blue colour. Now the tubes are cooled and the OD is read at 600nm.

AMPLIFICATION OF TMR GENE: Chromosomal DNA isolated from dye degrading organism was used as the template for PCR.

AGAROSE GEL ELECTROPHORESIS

The PCR products were mixed with loading dye (10 of sample in 3µl of bromophenol blue).

Agarose gel was prepared by taking 30 µl of 10X TEB and 0.6 g of agarose. The mixture was heated for 1-5 minutes for dissolve the agarose powder.To that add 2-5 µl of ethedium bromide was added and mixed properly. The mixture was poured in a gel mould scaled prior with comb and allowed to solidify.The samples were run till half the distance of gel and the bands were observed under uv-trans illuminator.

AMPLIFICATION: Amplify the product according to the standardized conditions. It is necessary to include final extension of 15 minutes to ensure the addition of 3'-A by Taq DNA polymerase. Note the addition of 3'-A is dependent on 5'- end base of primer. (Primers with 5'-C/G are known to yield maximum 3'-An additions).

The PCR product has to be analyzed on an agarose gel and quantified before setting up ligation. There should not be smearing, primer-dimer or additional bands present. If the template used for amplification is a plasmid, then the product has to be gel purified using silica/columns. This is to ensure that even trace amounts of plasmid template (used for amplification) is not present in sample as these plasmids also transform efficiently and obscure the result.

In general, removal of Taq polymerase and primers by phenol chloroform extraction and precipitation, column purification or by gel purification, improves cloning efficiency. (The purification has to be done with nuclease free reagents).

Ligation into T vector:

Thaw the T vector and control insert vials, centrifuge briefly to collect the contents at the bottom of the vials. Use 50 ng of the vector for each reaction and freeze the remaining vector at -20°C The control insert supplied with the kit is 600 bp PCR Product amplified from pBR322 & gel purified.

T4 DNA Ligase

Make up the volume to 10 µl with water. Mix the reaction components and give a short spin. Incubate at room temperature (25°C) for 1 hour. Freeze the ligation mixture till the time of transformation (if it is not possible to transform immediately) or continue ligation at 4°C

TRANSFORMATION:

Use high efficiency competent cells (minimum10^7 cfu/µg). The ligation of fragment with a single base pair over hang can be inefficient, so it is essential to use cells with high efficiency in order to obtain reasonable number of colonies. Transform competent cells with uncut pUC18 in parallel to ensure proper efficiency.To obtain sufficient number of clones, use competent cells with a minimum transformation efficiency of 10^7 cfu/µg.

Screening & Analysis

Inoculate single white colonies (10-20 Nos.) into 5 ml LB medium containing 100 µg/ml of ampicillin and 40µg/ml X-gal. Incubate overnight on shaker at 37°C.Carry out plasmid preparation and analyze by restriction digestion / sequencing.

Isolation of plasmid DNA:

Transfer a single bacterial colony in to 2 ml LB broth containing ampicillin antibiotic. Incubate the culture over night at 37^0c with vigorous shaking. Transfer 1.5 ml of the culture into microfuge tube. Centrifuge at 12,000 rpm for 30 secs at 4^oc in a microfuge. Store the remainder at 4^0c. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

Rinse the pellet of DNA with 1 ml of 70% ethanol a 4^0c. Remove the supernatant as described and allow the pellet of nucleic acid to dry in the air for 10 min. the pellet could be dried in vacuo. Redissolve the nucleic acid pellet in 50 µl of TTE (pH-8.0) containing DNase free pancreatic RNase (20 µl). Keep at 37^oc for 1 hr and then store the DNA at 4^0c.

RESULT AND DISCUSSION

We have isolated a bacterial strain Pseudomonas, which could degrade malachite green upto-80 µM. The activity of triphenylmethane reductase enzyme responsible for decolorizing triphenylmethane dyes was estimated. The enzyme involved in the degradation was estimated. A gene encoding the enzyme was amplified based on its N-terminal and internal amino acid sequence.Textile industries consume large volumes of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in chemical composition ranging from inorganic compounds to polymers and organic products (Mishra and Tripathy, 1993; Juang et al., (1996).

The presence of very low concentrations of dyes in effluent is highly visible and undesirable (Nigam et al., 2000). There are more than 100,000 commercially available dyes with over 700,000 ton of dyestuff produced annually (Meyer, 1981). The majority of the synthetic food colors have mono or triphenylmethane types (Morales-Álvarez et al 2018).

Triphenylmethane dyes include FD colors and C colors approved for use in USA and a number of delisted food crops were tested (Brown et al; 1978) in the Salmonella / Micro some system.

Fig 1	Fig 2
	
Fig 3	Fig 4

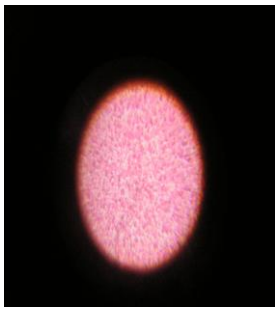
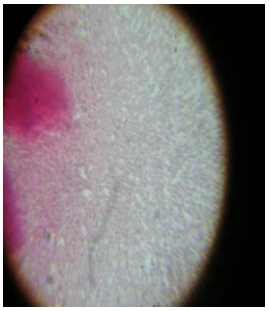

	

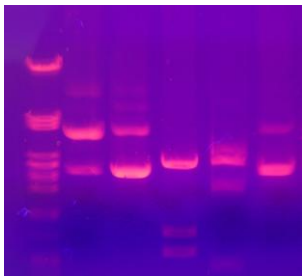
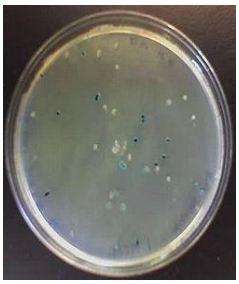
Fig 5	Fig 6
	

Table 1: **DISCOLORATION ASSAY**

S.No	Conc. of M.G(µM)	OD values at 600 nm	% Lalnunhlimi of discoloration	
		B	T	
1	20	1.58	1.38	12.6
2	40	1.68	1.50	10.7
3	60	1.74	1.76	1.14
4	80	1.83	1.82	0.54

GRAM NATURE OF PURE CULTURE ISOLATE: Table-2

Dye	Organism	Gram character
Isolate 1	*Pseudomonas*	Gram negative
Isolate2	*Pseudomonas*	Gram negative
Isolate 3	*Pseudomonas*	Gram negative

IMVIC RESULTS FOR THE 3 ISOLATES: Table-3

S. No	GRAM NATURE	INDOLE PRODUCTION	METHYL RED REDUCTION	VOGES PROSKAUER	CITRATE UTILIZATION	OXIDASE
Is 1	Gram negative *Pseudomonas*	-ve	-ve	-ve	+ve	+ve
Is2	Gram negative *Pseudomonas*	-ve	-ve	-ve	+ve	-ve

Is 3	Gram negative *Pseudomonas*	-ve	-ve	-ve	+ve	-ve

Table-4

Con of Malachite green(µM)	Optical Density at 600nm	Enzyme Activity(µ/min/ml)
20	0.44	0.0029
40	0.20	0.0014
60	0.19	0.0013
80	0.15	0.0010

Dye industries use the activated sludge process to treat effluent. However TPM dyes are toxic to the microbes and lessen their purifying action (Mahmoud et al 2017). Dye industries very frequently change the kind of dyes it is important for the treatment process by the acclimatization microbes to maintain their adaptation to different kind of dyes

The oxygen uptake rates of microbes, acclimatized through continuous culture in a medium containing dyes were obtained for the same type and different type of co-existing dyes and the influence of these dyes were investigated by Ogawa et al., (1981).

The ratio indicated that the growth inhibition strongly depends on the rate of RNA synthesis. The content ratios of the nucleic acids (RNA/DNA); decreased with the increasing dye concentration. These dyes act more preferentially to lower protein synthesis that inhibits cell division.

Due to the inhibitive action; cell shape varied, cells growing under ordinary conditions appeared as small rods and those in the presence of dyes, as filaments. It noted that dye inhibits DNA synthesis by stabilizing the double helix and by inhibiting the enzyme activities. 87 dyestuffs were tested in short-term aerobic biodegradation tests (Brown, 1986) to investigate whether some dyes might be susceptible to aerobic biodegradation and, if so, to what extent this occurs? For this work the dyestuff chosen were typical commercial products and bacteria inoculated were from effluent treatment plant (Muhd Julkapli et al 2014).

As in the static test, the criteria for biodegradation were both decolorization at the absorption maximum and dissolved organic carbon elimination. Their results confirmed that dye

stuffs are most unlikely to show any significant biodegradation in such tests.Many organisms are reported to decolorize various triphenlymethane and azo dyes. There are a few reports on the biodegradation of theses dyes by bacteria. In 1981 Yatome et al., reported the biodegradation of triphenylmethane dyes by Pseudomonas pseudomallei 13NA.

In general the decolorization of the dyes is not related to their molecular weights and the octanol- water coefficients of the dyes. Yatome et al., (1991) again reported the degradation of Crystal violet, Pararosaniline and Victoria growing cells of B.subtilis IFO 13719.

Biodegradation of triphenylmethane dyes by bacteria, fungi and yeasts (Azmi et al., 1998). They showed the advantages of using biological processes for degradation of dye molecules to carbon dioxide and water and with concomitant formation of less sludge and being eco-friendly.

Decolorization of triphenylmethane dyes and textile and dyestuff effluent by Kurthia sp. (Sani et al., 1999 , shah et al 2013). They screened a number of soil and water samples and isolated the Kurthia species on the basis of rapid dye decolorization. Under aerobic conditions 98% of the color was removed intracellular by this strain. A number of dyes such as crystal violet, malachite green. Ethyl violet etc was used for the studies.

After the decolorization of most of the dyes, viable cell concentration of Kurthia sp. reduced significantly (Lalnunhlimi et al 2016). Compared to crystal violet higher concentrations of malachite green was decolorized by the same amount of cell mass which may be due to the difference in the structure of both the dyes.

The first report of decolorization of triphenylmethane dyes (Yatome, 1991) by two Actinomycetes, Nocardia corallina and N. globerulla showed that decolorization activity is intracellular as there was no activity in the culture filtrate. The dyes were completely decolorized in 24 hours. They also detected degradation product of malachite green digestion as Michler's Ketone (MK) by N. globerulla. The decolorization activity was also not observed in the washed cells of N. corallina, when the cells were incubated in buffer but the activity regained when the cells were incubated in LB medium along with the product of biodegradation with Michler's ketone(Rawat et al , 2016)

Decoloration of triphenylmethane dyes (Crystal violet, Bromophenol blue and Malachite green) by three birds nest fungi – Cyathus bulleri, C. stercoreus, C. striatus was reported by (Vasdev et al., 1995.) Among the three organisms, C. buleri was found to be the most efficient in decolorization

Microbial decolorization and degradation is an environmentally friendly and cost-competitive alternative to chemical decomposition processes. To develop an efficient dye degradation biotechnology, the key step is to obtain broad-spectrum and highly efficient dye-decolorizing bacteria.Although many dye-decolorizing microorganisms have been reported (Banat et al. 1996; Azmi et al. 1998;) with exception of the decolorization of dyes by

Pseudomonas pseudomallei 13NA and Citrobacter sp. which decolorize both triphenylmethane and azo dyes by a single species of bacterium. However, though several microorganisms may seem to have a potential for dye degradation, very few strains can withstand the conditions of dyeing effluents (Maeir et.al 2004).

Olukanni et.al 2006 have discovered textile effluent adapted strains of Acinetobacter and Bacillus and effluent non adapted Bacillus species with potential use in the effluent treatment (Varma et al 2011) .Triphenyl methane dyes are decolorized by lignin peroxidase of Phanerochates chrysosporium (Bumps et.al 1988). Laccase from the extracellular fluid of Cyathus bullei (Vasdev et.al 1995) and peroxidase from Pleurotus ostreatus (Shin, K.S et.al 1998) also decolourise triphenyl methane dyes.

The structural genes encoding lignin peroxidase and laccase have been cloned and characterized (Cullen, D. 1997, Gold, M.H et.al 1993) although several triphenylmethane dye decolourising bacteria have been isolated (Azmi, W et.al 1998 & Sriram et al 2013) there are no reports of specific enzyme that decolourise these dyes. The decolorization of malachite green by intestinal microflora and several anaerobic bacteria proceeds their enzymatic reduction to their respective leuco derivatives (Henderson, A.I. et.al 1997, Mc Donald, J.J. et.al 1984). Moon-Sun Jang. Et.al 2005 isolated a new bacterium Citrobacter species and biochemically purified and characterized an enzyme that decolorizes triphenylmethane dyes (Bhattacharya et al 2018, subhathra et al 2013). They have designated the enzyme as triphenylmethane reductase(TMR).

CONCLUSION:

We have isolated a Pseudomonas strain from the textile dye effluent which could decolorize malachite green. The activity of triphenylmethane reductase enzyme responsible for decolorizing triphenylmethane dyes was estimated. The gene coding for triphenylmethane reductase was amplified and submitted for sequencing.

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