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CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research Vol. 8, Issue, 6, pp. 17628-17633, June, 2017 International Journal of Recent Scientific Re*r*earch

DOI: 10.24327/IJRSR

Research Article

STUDY ON DECOLORIZATION OF BROMOPHENOL BLUE AND CRYSTAL VIOLET BY ISOLATES PSEUDOMONAS SP. AND SALMONELLA SP.

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DOI: http://dx.doi.org/10.24327/ijrsr.2017.0806.0394

ARTICLE INFO	ABSTRACT
Article History: Received 15 th March, 2017 Received in revised form 25 th April, 2017 Accepted 28 th May, 2017 Published online 28 th June, 2017 Key Words: Crystal violet, Bromophenol blue, Decolorization, soil isolates	Azo dyes constitute the largest and most versatile class of synthetic dyes used in the textile, pharmaceutical, food and cosmetic industries and represent major components in wastewater from these industrial dying processes. The present investigation includes study of bacterial isolates from water samples collected from three sources <i>viz.</i> , Industry, Lake and Sewage. The bacterial isolates were then screened for the dye decolorizing ability in Mineral salt media (MSM) broth amended with 5ppm of Crystal violet and Bromophenol blue. Bacterial isolates G and H were found capable of degrading both Crystal violet and Bromophenol blue dyes to a greater extent within 120 hours of
	incubation. The decolorization potential of the two isolates G and H were investigated at different operational parameters like, Dye concentration, pH, Temperature, Carbon sources and Nitrogen
	sources. Among two isolates, isolate G showed considerably high percentage decolorization of Crystal violet and Bromophenol blue at 50ppm, 100ppm and 150ppm repectively. The isolates, G and H showed high decolorization at pH 8 and 5.6 within 120 hours of incubation for Crystal violet and Bromophenol blue. The isolate G and H were identified as Pseudomonas sp. and Salmonella sp. respectively based on morphological and biochemical characterization. The present investigation revealed these isolates can be used for treatment of effluents contaminated with Crystal violet and Bromophenol blue.

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INTRODUCTION

The earth once considered, to have an unlimited abundance of land and resources now shows a great depletion in the resources due to the human interference and negligence in using them. The problems associated with the contaminated sites now assume increasing prominence in many countries (Kochler and Kumar, 2012). Devising effective methods for controlling environmental pollution is presently one of the top most priorities in the field of scientific research.

One of the major reasons contributing to environmental pollution is the tremendous increase in industrial development (Puvaneswari *et al.*, 2006). Effluents containing colored dyes are released from leather, dyestuff, textile, food and dyeing industries. Effluents contaminated with dyes are majorly released by the textile industries (Lalnunhlimi and Krishnaswamy, 2016). The residual dyes released from these effluents introduce different organic pollutants in the natural water resources and land causing significant environmental

problems intern pose health hazards to human due to their toxicity. Therefore, remediation of these effluents is necessary to eliminate the risk to humans and environment.

Biodegradation is efficient method that offers decontamination and renders the various contaminants harmless using biological activity. Biodegradation is defined as the breakdown of complex organic substances into nontoxic or less toxic by utilizing living organisms or other biological means (Wang *et al.*, 2012).

The usage of Triphenylmethane dyes constitutes about 30-40% of total consumption of dyes (Ali *et al.*, 2009). Triphenylmethane dyes are aromatic xenobiotic compounds. Triphenylmethane dyes include Basic fuchsin, Crystal violet, Malachite green, Methyl violet, Ethyl violet, Methyl green, Brilliant green and Bromophenol blue etc.

Triphenylmethane dyes enter human body through food chain (Puvaneswari *et al.*, 2006). They are believed to be carcinogenic and mutagenic to humans and show high toxicity

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to mammalian cells (Hima Bindu Nidadavolu *et al.*, 2013). Use of triphenylmethane dyes has been banned in several countries due to their antagonistic effect on human health. Malachite Green is extremely toxic, acts as a potent carcinogen and adversely affects the human reproductive and immune systems (Ali *et al.*, 2009).

There are limited studies carried out on decolorization of Crystal violet and Bromophenol blue using bacterial isolates. Hence, the present study focuses on isolation of bacteria capable of decolorizing Crystal violet and Bromophenol blue and determining the effect of operational parameters on dye decolorization.

MATERIALS AND METHODS

Sample Collection

Samples were collected in clean, sterile and properly labeled 250 ml polystyrene bottles and transported aseptically to the lab for isolation of dye degrading microorganisms within 2 hours without affecting the viability of the samples and were Stored at 4°C un till further analysis. Samples were labeled as follows Sample I- Industrial water sample (IWS)

Sample II - Sewage water sample (SWS) Sample III- Lake water sample (LWS)

Isolation, purification, cultivation

After bringing samples to lab temperature the samples were inoculated in nutrient medium of pH 7 and 37°C for 24 hour incubation. 0.1 mL aliquot of serially diluted wastewater samples were spread plate on to nutrient agar plates. After 24 hour incubation, morphologically distinct colonies were picked, purified by streak plate method and maintained by sub culturing them on nutrient agar slants stored at 4°C.

Screening of bacteria for dye decolorizing ability

Loopful of culture from nutrient slant was inoculated into 50 mL test tubes containing 12 mL of Mineral salt medium broth (MSM). Two separate sets of MSM broth were prepared first set was supplemented with (5ppm) of crystal violet dye and second set was supplemented with (5ppm) of bromophenol blue dye.

Identification of dye - decolorizing bacteria

Dye degrading bacteria were identified using colony morphology, Gram staining and biochemical characterization. Gram staining is based on the differences in the cell walls of Gram-positive and Gram-negative organisms. A thin smear of suspension was prepared on a clean glass slide allowed to air dry, and heat fixed then the smear was flooded with Crystal violet for 60 seconds. The slide was rinsed with distilled water and covered with Gram's iodine for 60 seconds. The smear was immediately decolorized with 70 % Ethyl alcohol. Later counter stained with safranin for 30 seconds the slide was rinsed with distilled water, blot dried and examined under the oil immersion lens of a microscope (Sharma, 2007).

Biochemical Characterization

Morphological and Biochemical characterization of soil isolates was done by Gram staining, endospore staining followed by biochemical tests Indole Test(I), Methyl Red

(MR), Test Voges-Proskauer (VP) Test, Citrate Utilization (C) test Carbohydrate fermentation Tests (Phenol Red Broth) Catalase test (Sharma, 2007).

Decolorization assay

2mL of 24 hour bacterial culture was inoculated into 250mL Erlenmeyer's flask containing 100mL of Mineral salt medium broth and incubated at 30°C for 24hours. After 24hours incubation dye was added at concentration of 50ppm. 5mL aliquot of culture media was withdrawn at different time intervals and centrifuged at 10,000 rpm for 15 minutes to microbial separate the cells. Using UV-Visible spectrophotometer absorbance of clear supernatant was measured at the absorption maxima of respective dyes and plotted absorbance versus time curves to show removal of the dye (Ogugbue and Sawidis, 2011).

Decolorization activity was calculated using formula

Decolorization (%) = <u>Initial absorbance – Observed absorbance</u>
Initial absorbance

Decolorization studied at different operational parameters

Bacterial isolates showing high decolorizing capacity were further studied to investigate the effect of various operational parameters on the dye decolorization, using various dye concentrations (50ppm,100ppm,150ppm), pH (5.6,7.0, 8.0), temperature (RT, 37°C, 40°C), 1% of carbon sources (glucose, fructose, lactose) and Nitrogen sources (tryptone, beef extract, peptone). Dye decolorization was monitored using UV -Visible Spectrophotometer and the percent decolorization was determined.

RESULTS

The decolorization potential of two isolates *viz.*, Isolate G and H were studied under different operational conditions dye concentration, pH, Temperature, Carbon and Nitrogen sources. Two triphenylmethane dyes *viz.*, Crystal violet and Bromophenol blue were used for the study. Varying percentage of decolorization was reported under different operational conditions. The results of the decolorization experiments were expressed in terms of percent decolorization by determining the difference between the initial and final stage of observation using UV-Visible spectrophotometer.

Isolation and Screening of bacterial isolates

The selective enrichment of water samples collected from three different sources viz., Industry, Sewage and lake led to the isolation of 14 morphologically different bacterial isolates (Table 1). Gram staining of all isolates indicated the presence of 2 Gram positive and 12 Gram negative organisms. The pure cultures were preserved on nutrient agar medium at 4°C. All 14 isolates decolorized both the dyes with different capacity ranging from lowest (41.1%) to highest (88.2%) in case of Crystal violet (Table 3) and lowest (5%) to highest (70%) in case of Bromophenol blue (Table 4).

The isolate G colonies were white, butyrous, and opaque, lobate with irregular margins and were observed to be Gram negative rods. The isolate H colonies were white, butyrous, opaque, entire with irregular margins and were observed to be Gram negative rods.

Isolates	Size	Pigmentation	Formation	Margin	Elevation	Opacity	Consistency
IWS-A	Large	Pink	Rhizoid	Lobate	Raised	Opaque	Dry
IWS-C	Moderate	No pigmentation	Irregular	Serrate	Raised	Opaque	Butyrous
IWS-D	Small	White	Circular	Entire	Flat	Translucent	Dry
SWS-E	Large	White	Rhizoid	Lobate	Raised	Translucent	Butyrous
SWS-F	Large	White	Irregular	Serrate	Raised	Opaque	Butyrous
SWS-G	Large	White	Irregular	Lobate	Raised	Opaque	Butyrous
SWS-H	Small	White	Circular	Entire	Raised	Opaque	Butyrous
SWS-I	Small	No pigmentation	Circular	entire	Raised	Opaque	Butyrous
LWS-J	Small	No pigmentation	Circular	Entire	Raised	Opaque	Slimy
LWS-K	Small	Pink	Circular	Entire	Raised	Opaque	Butyrous
LWS-L	Small	No pigmentation	Circular	Entire	Raised	Opaque	Butyrous
LWS-M	Small	No pigmentation	Circular	Entire	Raised	Translucent	Slimy
LWS-N	Small	Milky	Irregular	Lobate	Flat	Translucent	Butyrous

Table 1 Morphological Ch	aracteristics of isolates
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Based on the morphological and biochemical characteristics Isolate G and isolate H were identified as *Pseudomonas* sp. and as *Salmonella* sp respectively (Table 2). decolorization which decreased to 61.3% at 100ppm, further, decreased to 34.8% at 150ppm of Crystal violet.

Sr. No	Isolates	Indole Test	MR Test	VP Test	Citrate Test	Glucose fermentation test	Catalase test
1.	SWS-G	-	-	-	-	-	-
2.	SWS-H	-	+	-	-	-	-

(+ : Positive result, - :Negative result)

Effect of Dye concentration

Decolorization property of the organisms was greatly influenced by the dye concentration. The study has been carried out with 2 dyes Crystal violet and Bromophenol blue at increasing concentrations of 50ppm, 100ppm, and 150ppm. However, the isolates exhibited varying Decolorization patterns.

Table 3 Decolorization Percentage for Crystal violet

Isolates	Dye	Optical density at 590 nm	Decolorization %
IWS-A		0.05	70.5
IWS-C		0.04	76.4
IWS-D		0.04	76.4
SWS-E		0.04	76.4
SWS-F	let	0.04	76.4
SWS-G	/io	0.02	88.2
SWS-H	Crystal Violet	0.02	88.2
SWS-I	/sta	0.04	76.4
LWS-J	5	0.07	58.8
LWS-K		0.04	76.4
LWS-L		0.06	64.70
LWS-M		0.10	41.1
LWS-N		0.04	76.4

Table 4 Decolorization percentage for	Bromophenol blue
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Isolates	Dye	Optical density at 598 nm	Decolorization %
IWS-A		0.11	45
IWS-C		0.09	55
IWS-D		0.14	30
SWS-E	ue	0.10	50
SWS-F	Bromophenol blue	0.11	45
SWS-G	lou	0.06	70
SWS-H	hei	0.07	65
SWS-I	dor	0.13	35
LWS-J	no	0.12	40
LWS-K	Br	0.15	25
LWS-L		0.17	15
LWS-M		0.18	10
LWS-N		0.19	5

decreased to 37% at 100ppm and further decreased to 22.4% at 150ppm of Bromophenol blue. The study also revealed that at 50ppm concentration, isolate H showed 71% decolorization and decreased to 65% at 100ppm, further, to 48% at 150ppm of Crystal violet. Isolate H showed 54% decolorization at 50ppm and decreased to 30% at 100ppm, further to 21.4% at 150 ppm of Bromophenol blue (Table 5 and Table 6).

Effect of pH

The decolorization potential of the three isolates B, G and H were investigated at 3 different pH values 5.6, 7 and 8.Isolate G showed decolorization of 95%, 88% and 100% at pH 5.6, pH 7 and pH 8 respectively for 50 ppm of Bromophenol blue within 120 hour incubation. Isolate H showed decolorization of 59.3%, 78.1% and 87.5% at pH 5.6, pH 7 and pH 8 respectively for 50 ppm of Crystal violet within 120 hour incubation. Isolate H showed decolorization of 97%, 93% and 97.6% at pH 5.6, pH 7 and pH 8 respectively for 50 ppm of Crystal violet within 120 hour incubation. Isolate H showed decolorization of 97%, 93% and 97.6% at pH 5.6, pH 7 and pH 8 respectively for 50 ppm of Bromophenol blue within 120 hour incubation (Table 5 and Table 6).

Effect of Temperature

The decolorization potential of the three isolates B, G, H was investigated at three different temperatures Room temperature (RT-33°C), 37°C, 40°C. Isolate G showed decolorization of 71%, 68% and 71% at RT, 37°C and 40°C respectively for 50ppm of Crystal violet within 120 hours incubation. Isolate G showed decolorization of 70.7%, 73% and 70.7% at RT, 37°C and 40°C respectively for 50ppm of Bromophenol blue within 120hours incubation.Isolate H showed decolorization of 71%, 65% and 48% at RT, 37°C and 40°C respectively for 50ppm of Crystal violet within 120hours incubation. Isolate H showed decolorization of 71%, 65% and 48% at RT, 37°C and 40°C respectively for 50ppm of Crystal violet within 120hours incubation. Isolate H showed decolorization of 73%, 68.2% and 63.4% at RT, 37°C and 40°C respectively, for 50ppm of Bromophenol blue within 120hours incubation (Table 5 and Table 6).

Culture media			I				(%) ¢	lecoloriz	ation			
	Isolate	dye	Incubation time	Dye concentration(ppm) Temperature(°C) pH						pН		
		-	time	50	100	150	RT	37	40	5.6	7.0	8.0
Mineral Salt	SWS-G			91.6	61.3	34.8	71	68	71	65.6	81.25	90.6
Medium (MSM)	SWS-H	CV	5 Days	80.5	57.3	35.8	71	65	48	59.3	78.1	87.5

Table 5 Decolorization percentage of Crystal violet at varying Dye concentration, Temperature and pH

(CV- Crystal violet RT-Room temperature)

Table 6 Decolorization percentage of Bromophenol blue at varying Dye concentration, Temperature and pH

Culture media							(%)	decoloriz	ation			
	Isolate	dye	Incubation time	Dye	Dye concentration (ppm) Temperature(°C) pH				рН			
				50	100	150	RT	37	40	5.6		8.0
Mineral Salt	SWS-G			64.2	37	22.4	70.7	73	70.7	95	88	100
Medium (MSM)	SWS-H	BB	5 Days	54.1	30	21.4	73	68.2	63.4	97	93	97.6

(BB-Bromophenol Blue RT-Room temperature)

Effect of Carbon source

The dye decolorization efficiency of the bacterial isolates was monitored with different 1% carbon sources supplemented in the MSM broth. Carbon sources used in the study were Glucose, Fructose, and Lactose. Isolate G showed decolorization of 65%, 36.3% and 68.1% for Glucose, Fructose and Lactose respectively at 50ppm of Bromophenol blue within 120 hours incubation. Isolate H showed decolorization of 85.7%, 60% and 66% with Glucose, Fructose and Lactose respectively at 50ppm of Crystal violet within 120 hours incubation. Isolate H showed decolorization of 59%, 34% and 75% with Glucose, Fructose and Lactose respectively at 50ppm of Bromophenol blue within 120hours incubation (Table 7 and Table 8). Isolate H showed decolorization of 59%, 65.7% and 82.8 % for Beef extract, Peptone and Tryptone respectively for 50ppm of Crystal violet within 120hours incubation. Isolate H showed decolorization of 68% and 64.4% for Peptone and Tryptone respectively at 50ppm of Bromophenol blue within 120 hours incubation (Table 7 and Table 8).

DISCUSSION

The decolorization potential of the isolates G and H against the dyes Crystal violet and Bromophenol blue were discussed with reference to relevance of earlier studies.

Table 7 Decolorization percentage of Crystal violet with Different Carbon and Nitrogen Sources

Culture media				(%) decolorization						
	Isolate	dye	Incubation time	(19	Carbon source % Concentration		Nitrogen source (1% Concentration)			
		·		Glucose	Fructose	Lactose	Beef extract	Peptone	Tryptone	
Mineral Salt	SWS-G			90.9	63	60	54	77	74.2	
Medium (MSM)	SWS-H	CV	5 Days	85.7	60	66	59	65.7	82.8	

 Table 8 Decolorization percentage of Bromophenol blue with Different Carbon and Nitrogen Sources

Culture media	Isolate	dye	Incubation time	(%) decolorization					
				Carbon source (1% Concentration)			Nitrogen source (1% Concentration)		
				Glucose	Fructose	Lactose	Beef extract	Peptone	Tryptone
Mineral Salt	SWS-G			65	36.3	68.1	-	64.4	68.8
Medium (MSM)	SWS-H	BB	5 Days	59	34	75	-	68	64.4

Effect of Nitrogen sources

The decolorization potential of the bacterial isolate was monitored with different Nitrogen sources at concentration of 1% each, supplemented in the MSM broth. Nitrogen sources used in the study were Beef extract, Peptone, Tryptone. Isolate G showed decolorization of 54%, 77% and 74.2% for Beef extract, Peptone and Tryptone respectively at 50ppm of Crystal violet within 120hours incubation. Isolate G showed decolorization of 64.4% and 68.8 %, at Peptone and Tryptone respectively at 50ppm of Bromophenol blue within 120 hours incubation.

Effect of dye concentration

The dye concentration can affect the decolorization efficiency especially due to toxicity of dye at higher concentrations. Decolorization of 78% was obtained for Crystal violet, respectively, at 50mg/L. Decrease in decolorization percentage was observed with increase in dye concentration (Ogugbue and Sawidis, 2011). High dye concentration showed toxic effect that adversely affected decolorization (Saratale *et al.*, 20060. Radhika *et al.*, (2014) studied decolorization of Bromophenol blue using fungal cultures which reported that decolorization

percentages decreased with increase in dye concentrations. In the present study it was observed that high dye concentration acted as an inhibitor for bacterial growth and resulted in less decolorization. In the present study it was observed that isolates G and H at 150ppm of Crystal violet showed dye decolorization of 34.8% and 35.8%. Whereas, at 150ppm of Bromophenol blue, Isolates G and Hshowed dye decolorization of 22.4% and 21.4% respectively. Isolate G and H at 50ppm of Crystal violet showed high decolorization of 91.6% and 80.5%. Similarly, high decolorization of 64.2% and 54.1% for Bromophenol blue was observed. Results of present are in concurrence with earlier studies. Where, higher decolorization was observed at lower dye concentrations.

Effect of pH

It has been reported that the optimum pH for decolorization is often at neutral pH value or a slightly alkaline value (Pearce *et al.*, 2003). Ogugbue and Sawidis, (2011) have reported that the optimum pH for color removal is at a slightly alkaline pH. The optimum pH for dye decolorization was found to be at pH 7-8. In the present study, the isolates, G and H showed highest decolorization of 90.6% and 87.5% respectively for Crystal violet at pH 8. Also, the isolates G and H showed highest decolorization of 100% and 97.6% respectively for Bromophenol blue at pH 8.The isolates showed highest decolorization at a slightly alkaline pH. Similar, results were observed in earlier studies. Where, efficient decolorization was observed at higher pH values.

Effect of Temperature

Many studies have reported that with increase in temperature the rate of decolorization increases. The optimum temperature for color removal tends to correspond with the temperature for optimum cell growth i.e., (35°-45°C). Ogugbue and Sawidis, (2011) have reported that optimum temperature required for dye decolorization was found to be 35°C. In the present study, isolates G and H showed high decolorization of 71% and 71% respectively, at 50ppm of Crystal violet at 33°C. Whereas, the isolates G and H showed high decolorization of 70.7% and 73% respectively, at 50ppm of Bromophenol blue at 33°C. Similar findings were reported in earlier studies where, incubation temperature 35°C showed more efficient decolorization at incubation temperatures of 33°C (RT) and 37°C.

Effects of Carbon and Nitrogen sources

Effects of several carbon and nitrogen sources on bacterial decolorization performance have been studied in previous findings. Presence of nitrogen and carbon sources has proved to enhance bacterial decolorization. In the presence of 1% nitrogen sources (urea, peptone and malt extract) the culture exhibited 87.5, 81.25 and 38.3% (Parshetti *et al.*, 2010). Presence of molasses, urea and sucrose in synthetic medium showed 100% of decolorization (Parshetti *et al.*, 2006). In the present study, Isolate G and H showed decolorization of 90.9% and 85.7% respectively at 50ppm of Crystal Violet when 1% Glucose was supplemented as carbon source. Isolate G showed decolorization of 77% for Crystal violet when Peptone was used as Nitrogen source. It was observed that supplementing medium with carbon and nitrogen sources has enhanced the

decolorization efficiency of the bacterial isolates, which is similar to the earlier findings.

CONCLUSION

The reports available on biological decolorization of wastewater containing Triphenylmethane dyes are limited. In the present study, we describe the isolation and screening of bacterial isolates capable of efficiently removing triphenylmethane dyes from waste water. From this study, we conclude, that the three bacterial isolates G and H showed high decolorization efficiency for dyes Crystal Violet and Bromophenol blue. Dye decolorization by these isolates was found to be dependent on dye concentration, pH, Temperature and presence of carbon and nitrogen sources.

In order to gain a clear insight into the decolorization mechanism employed by the bacterial isolates, there is need to identify and purify the enzymes produced by bacterial isolates which are involved in degradation of the dyes.

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How to cite this article:

Potadar R. R *et al.*2017, Study on Decolorization of Bromophenol Blue and Crystal Violet by Isolates Pseudomonas sp. and Salmonella SP. *Int J Recent Sci Res.* 8(6), pp. 17628-17633. DOI: http://dx.doi.org/10.24327/ijrsr.2017.0806.0394

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