**Characterization and Optimization of Triphenylmethane dye 'Brilliant Green' Degradation by four indigenous bacterial species**

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**Abstract:** Dye wastes represent one of the problematic groups of pollutants because their presence can easily be identified by the human eye and they are not easily biodegradable. Brilliant green is a Triphenylmethane dye with known genetoxic and carcinogenic properties. The dye is commonly used in paper and pulp mill industry and is discharged into nearby water bodies as coloured wastewater. In this study an attempt was made to investigate the optimum parameters of Brilliant green decolourization by four selected potential organisms isolated from discharged effluents. During the study of incubation condition of Brilliant green decolourization, shaking condition was found to be more effective than the static condition. Out of the four isolates *Micrococcus luteus and Bacillus licheniformis* showed 100% decolourization of Brilliant green after 3 days in shaking condition. The optimum temperature range for the maximum decolourization of Brilliant green was 30-37$^0$C. All of our selected isolates were found to exhibit a higher decolourization rate at pH near 7.0. The best decolourization was achieved at pH 7.0 with 87% decolourization by *Bacillus licheniformis*. *Planococcus citreus*, *Micrococcus luteus* and *Bacillus licheniformis* exhibited higher decolourization activity when glucose was used as a co-substrate in Normal decolourization medium. The decolourization rate by these organisms was 85%, 91% and 91% respectively. The use of starch as a co-substrate enhanced the growth of *Bacillus brevis*. This organism decolourized Brilliant green about 75% after 3 days incubation in the presence of starch. The four isolates were found to show higher decolourization in organic supplement containing modified broth than in inorganic modified broth. *Micrococcus luteus* showed 80% decolourization of the dye in organic condition.

**Keywords:** Absorbance, Brilliant green, Decolourization, Triphenylmethane, Wastewater

## INTRODUCTION

A dye can generally be described as a colored substance that has an affinity to the substrate to which it is being applied. Azo and triphenylmethane dyes are the largest classes of commercially produced colourants. Azo dyes are characterized by the presence of chromophoric azo group and triphenylmethane dyes by the presence of chromogen containing three phenyl groups bound by the central carbon atom **(Table 1)**. They are used for colouring paper, food, cosmetics, textiles, leather and in medical treatment and analysis (Swamy and Ramsay 1999; Somasiri *et al*. 2006; Padamavathy *et al.* 2003).

Brilliant Green is one of the important dyes of Triphenylmethane group. Triphenylmethanes - Malachite Green (MG), Crystal Violet (CV) and Brilliant Green (BG) are dyes with known genotoxic and carcinogenic properties. Apart from being illegally used in aquaculture for treatment of fish diseases they are also applied in industry such as paper production to colour paper towels widely used in hospitals, factories and other locations for hand drying after washing. The consumption of fish contaminated with these dyes due to the illegal application in aquaculture is a high risk for human.

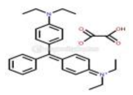
Losses of colourants during different stages of manufacture can reach even 10–15%. Coloured wastewaters, containing such high concentrations of dyes, lead to surface water pollution. A very small amount of dye in water (10-50 mg/ L) affects the aesthetic value, transparency of water and gas solubility of water bodies. Disposal of these dyes into the environment cause serious damage since they reduce light penetration and photosynthetic activity, cause deficiency of oxygen, are highly toxic and are mutagenic. Synthetic origin and complex aromatic structure make them more recalcitrant to biodegradation (Sani and Banerjee 1999; Azmi *et al.* 1998; Banat *et al.* 1996; Pointing and Vrijmoed 2000).

Dyes can be removed from wastewater by chemical and physical methods including adsorption, coagulation-flocculation, oxidation, electrochemical methods. However both the physical and chemical have many disadvantages in application, such as high energy cost, high-sludge production, formation of by-products (Sariogula *et al*. 2007). On the other hand bio-processing based on bio sorption, bioaccumulation and biodegradation can overcome these defects because it is cost saving and environmentally friendly. These processes use low-cost biological materials as living or dead microorganisms. Fungi (Acuner and Dilek 2004, Asgher *et al* 2008) and algae (Daneshvar *et al* 2007) have been used in dye decolourization. Bio adsorption rather than degradation plays a major role during decolourization process by fungi and algae. However, this process is similar to different physical adsorption mechanisms and is not suitable for long-term treatment. Consequently the dyes remain in the environment. Biotransformation of dye structure can lead to formation of less toxic products or complete mineralization (Azmi *et al.* 1998; Pointing and Vrijmoed 2000; Robinson *et al*.

2001). It is well known that bacteria can degrade and even completely mineralize many dyes under certain conditions (Asad *et al.* 2007, Chen *et al* 2003).

n the present study, an attempt has been made for the degradation and decolourization of a triphenylmethane dye using individual bacterial culture in flasks. Effect of various process parameters like pH, temperature, co-substrates and incubation condition of dye was studied.

**Table 1:** Properties of Brilliant green.

| Property | Brilliant Green |
|---|---|
| Solubility in water | 4% |
| Solubility in Ethanol | 5% |
| Absorption maximum | 428,625(Aldrich) |
| Colour | Green |
| Emperical formula | $C_{27}H_{34}N_2O_4S$ |
| Molecular Weight | 466.36 |
| Chemical Formula |  |

## MATERIALS AND METHODS

### Location of effluent sample collection

The effluent samples were collected from the different places of one of the biggest paper industries, located near Karnafully river in Chittagong, Bangladesh. The industry produces both white and coloured paper.

### Isolation and Presumptive selection of Brilliant green degrading organisms

For isolation of bacteria from collected effluents Nutrient agar medium was used. The effluent samples were serially diluted up to $10^6$ dilutions. For isolation pour plate method was carried out and then the plates were incubated at $37^0$C for 48 hours. After incubation the discrete bacterial colonies were purified by repeated streak plating method. The purified cultures were preserved at $4^0$C for further study.

For presumptive selection of Brilliant green degrading bacterial isolates Nutrient broth medium containing 50 mg/L of Brilliant green was dispensed into the test tubes (10 ml per test tube) and then autoclaved at $121^0$C for 15 mins. This modified broth was then inoculated with the individual freshly grown organism, isolated from the paper and pulp mill effluent and incubated at $37^0$C for 7 days. The modified broth without culture suspension was also maintained and considered as control. The decolourization of dye was observed visually by comparing with control periodically and recorded.

**Identification of selected bacterial isolates**

The bacterial isolates were characterized and identified by studying different properties including size and shape of the organisms, arrangement of the cells, presence or absence of spores, regular or irregular forms, acid fastness, Gram reaction; cultural and physiological characteristics including temperature, salt tolerance, IMViC test, $H_2S$ production, nitrate reduction, deep glucose agar test, fermentation of different carbohydrates and some other biochemical reactions. All these characteristics were then compared to the standard description of "Bergey's Manual of Determinative Bacteriology" 8$^{th}$ ed. (Buchanan and Gibbons 1987).

**Effect of different parameters on decolourization of Brilliant green**

The bacterial strains were prepared in the liquid medium (Nutrient broth) in the shake flasks at 30$^0$C, 200 rpm (orbital incubator Model SI50, Stuart scientific, UK) for 20h. Decolourization test was done by adding 5% (v/v) of the inoculum to the modified Normal decolourization medium (NDM) ($(NH_4)_2SO_4$ 0.25gm, Yeast extract 0.25gm, $KH_2PO_4$ 0.5gm, $MgSO_4.7H_2O$ 0.05gm, $CaCl_2.2H_2O$ 0.013gm, Starch/Glucose 2.0 gm, Distilled water 1000 ml) containing 50 mg/L of Brilliant green, pH adjusted to 6.0-7.5 with 0.1M phosphate buffer.

The bacterial suspension was inoculated in 250 ml Erlenmeyer flask containing 50 ml modified Normal decolourization medium to study the effect of shaking and static conditions on dye decolourization. Flasks were shaken at 200 rpm on the orbital shaker. Both static and shake flasks were established at 30$^0$C for 3 days.

Effect of different other parameters on dye degradation including variation of temperature (30$^0$C, 37$^0$C & 45$^0$C) and pH (4.5, 7.0 and 8.5), presence of co-substrates (glucose, starch and sucrose), inorganic and organic conditions on Brilliant green decolourization were investigated. To investigate the effect of inorganic environment on dye decolourization Mineral Salt Medium (MSM) ($NaH_2PO_4$ 0.235gm, $MgSO_4.7H_2O$ 0.07gm, $CaCl_2$ 0.014gm, $FeCl_3.6H_2O$ 0.001gm, Distilled water 1000 ml) containing 50 mg/L of dye was used. For each culture condition, control broth without adding culture suspension was maintained. One replicate sample was kept for each individual condition.

**Measurement of decolourization rate of Brilliant green**

Broth cultures were withdrawn after 3 days of incubation, centrifuged at 10,000g for 15 min at 10$^0$C and the supernatant was used for analysis.

Optical absorbance of the untreated and treated samples was measured at 520nm under visible light using a spectrophotometer. The rate of decolourization was calculated from the following formulae described by Sani and Banerjee (1999).

$$\text{Percentage of decolourization} = \frac{Initial\ absorbance - after\ decolourization\ absorbance}{Initial\ absorbance} \times 100\%$$

## RESULT AND DISCUSSION

### Isolation & Presumptive selection of the isolates

A total of number of 11 bacterial colonies were isolated and purified. Out of the 11 isolates four isolates were presumptively selected for their ability of Brilliant green decolourization. The four isolates were named as KT3, KT5, KT7 and KT11.

### Identification of the selected bacterial isolates

Identification of isolates was a sequential process and was done by studying their morphological, cultural (**Table 2**), biochemical and physiological properties. Finally four Brilliant green degraders were identified as *Planococcus citreus* (KT3), *Bacillus brevis* (KT5), *Micrococcus luteus* (KT7) and *Bacillus licheniformis* (KT11) by comparing with the standard description of "Bergey's Manual of Determinative Bacteriology" 8$^{th}$ ed. (Buchanan and Gibbons 1987).

**Table 2:** Cultural characters of the selected isolates on nutrient agar.

| No. of Isolates | Cultural Characters | | | | | Slant Characters |
|---|---|---|---|---|---|---|
| | Form | Colour | Elevation | Margin | Surface | |
| KT3 | Circular | Yellowish | Convex | Entire | Smooth | Echinulate |
| KT5 | Circular | Creamish white | Convex | Entire | Smooth | Echinulate |
| KT7 | Circular | Yellow | Convex | Entire | Smooth | Filiform |
| KT11 | Circular | Opaque to brownish | Convex | Entire | Smooth | Spreading |

**Effect of culture conditions on decolourization of Brilliant green**

Incubation of Brilliant green containing modified broth under shaking condition demonstrated a better rate of decolourization than that of under static condition. All of our selected isolates showed higher decolourization efficiency under shaking condition than the static condition (**Table 3**). Out of the four isolates *Micrococcus luteus* (KT7) *and Bacillus licheniformis* (KT11) showed 100% decolourization of Brilliant green after 3 days in shaking condition. These isolates showed 91.42% decolourization rate under static condition. This decolourization rate indicated the efficiency of decolourization by these two organisms under both conditions. This result suggested that oxygen was favourable to the growth of the organisms and it also serves as a terminal electron receptor over the triphenyl group.

**Table 3:** Effect of Brilliant green decolourization at static and shaking condition.

| Strain | Absorbance & Percentage of Decolourization | | | |
|---|---|---|---|---|
| | Static condition | | Shaking condition | |
| | Control | Treatment | Control | Treatment |
| *Planococcus citreus* | .35 | .05(85.71%) | .35 | .03(91.42%) |
| *Bacillus brevis* | .35 | .14(60.00%) | .35 | .10(71.42%) |
| *Micrococcus luteus* | .35 | .03(91.42%) | .35 | .00(100.00%) |
| *Bacillus licheniformis* | .35 | .03(91.42%) | .35 | .00(100.00%) |

**Effect of temperature on decolourization of Brilliant green**

The dye decolourization activity of our cultures was found to show a significant rate from 30$^0$C to 37$^0$C (**Table 4**). The decolourization rate decreased with the increase of incubation temperatures. Among the four isolates *Bacillus licheniformis* (KT11) showed mostly similar level of decolourization rate from 30$^0$C to 37$^0$C. Decolourization activity was significantly reduced at 45$^0$C for all of the organisms. Decline in decolourization activity can be attributed to loss of cell viability at higher temperature or to the denaturation of peroxidase enzymes (Cetin and Donmez 2006). However among the four isolates *Micrococcus luteus* (KT7) showed maximum rate of decolourization at 45$^0$C.

**Table 4:** Effect of temperatures on decolourization of Brilliant green.

| Strain | Absorbance & Percentage of Decolourization | | | | | |
|---|---|---|---|---|---|---|
| | $30^0C$ | | $37^0C$ | | $45^0C$ | |
| | Control | Treatment | Control | Treatment | Control | Treatment |
| *Planococcus citreus* | .32 | .05(84.37%) | .32 | .08(75.00%) | .32 | .13(59.37%) |
| *Bacillus brevis* | .32 | .13(59.37%) | .32 | .15(53.12%) | .32 | .20(37.50%) |
| *Micrococcus luteus* | .32 | .04(87.50%) | .32 | .08(75.00%) | .32 | .09(71.87%) |
| *Bacillus licheniformis* | .32 | .04(87.50%) | .32 | .06(81.25%) | .32 | .12(62.50%) |

**Effect of pH on decolourization of Brilliant green**

The pH plays vital role in decolourization activity of the triphenylmethane dye and the optimum pH for colour removal is often between 6.0 and 10.0 (Chen *et al* 2003; Guo *et al* 2007). Bacterial cultures generally exhibited maximum decolourization rate at pH values near 7.0. The decolourization rate of Brilliant green by our isolates was found to increase by increasing pH values from 4.5 to 7.0 **(Table 5)** and maximum decolourization was observed at pH 7.0. This could be indicated the fact that the optimum pH for the growth of our selected cultures was 7.0. The rate of decolourization was observed to decrease with increase of pH value from 7 to 8.5. The best decolourization was achieved at pH 7.0 with 87% decolourization by *Bacillus licheniformis* (KT11).

**Table 5:** Effect of pH on decolourization of Brilliant green.

| Strain | Absorbance & Percentage of Decolourization | | | | | |
|---|---|---|---|---|---|---|
| | pH 4.5 | | pH 7.0 | | pH 8.5 | |
| | Control | Treatment | Control | Treatment | Control | Treatment |
| *Planococcus citreus* | .26 | .10(61.53%) | .32 | .06(81.25%) | .23 | .15(34.78%) |
| *Bacillus brevis* | .26 | .16(38.46%) | .32 | .13(59.37%) | .23 | .17(26.08%) |
| *Micrococcus luteus* | .26 | .13(50.00%) | .32 | .05(84.37%) | .23 | .13(47.82%) |
| *Bacillus licheniformis* | .26 | .12(53.84%) | .32 | .04(87.50%) | .23 | .15(34.78%) |

**Effect of co-substrates on decolourization of Brilliant green**

*Planococcus citreus* (KT3), *Micrococcus luteus* (KT7) and *Bacillus licheniformis* (KT11) exhibited higher decolourization activity when glucose was used as a co-substrate in Normal decolourization medium. The same organisms showed lower decolourization activity in the presence of sucrose and starch (**Table 6**). N A Oranusi, C J Ogugbue found *Pseudomonas sp.* to decolourize Brilliant green about 93% in the presence of glucose as co-substrate. Enhanced decolourization was attributed due to generation of redox equivalent (electron receptor) as result of metabolism of glucose. The use of starch as co-substrate enhanced the biodegradation of Brilliant green by *Bacillus brevis* (KT5) in compared to the presence of glucose and sucrose. This indicated the preferential utilization of starch by this organism to generate useful metabolites as electron receptor and thus to enhance decolourization activity.

**Table 6:** Effect of different co-substrates on decolourization of Brilliant green.

| Strain | Absorbance & Percentage of Decolourization | | | | | |
|---|---|---|---|---|---|---|
| | Glucose | | Starch | | Sucrose | |
| | Control | Treatment | Control | Treatment | Control | Treatment |
| *Planococcus citreus* | .35 | .05(85.71%) | .28 | .10(64.28%) | .25 | .12(52.00%) |
| *Bacillus brevis* | .35 | .14(60.00%) | .28 | .08(71.42%) | .25 | .13(48.00%) |
| *Micrococcus luteus* | .35 | .03(91.42%) | .28 | .10(64.28%) | .25 | .09(64.00%) |
| *Bacillus licheniformis* | .35 | .03(91.42%) | .28 | .13(53.57%) | .25 | .09(64.00%) |

**Effect of inorganic and organic condition on decolourization of Brilliant green**

All of our isolates were found to show higher decolourization in organic supplement containing modified broth than the inorganic condition **(Table 7)**. The possible reason for higher decolourization is the generation of redox equivalent from glucose/starch by the organisms which acts as an electron receptor. The decolourization rate of Brilliant green by *Planococcus citreus* (KT3), *Bacillus brevis* (KT5), *Bacillus licheniformis* (KT11) in inorganic condition is mostly similar but they show variation when inoculated in organic medium. In normal decolourization medium two *Bacillus* species showed lower decolourization activity in compared to two other. Among the four isolates *Micrococcus luteus* (KT7) showed highest rate of decolourization of Brilliant green.

**Table 7:** Effect of inorganic and organic condition on decolourization of Brilliant green**.**

| Strain | Absorbance & Percentage of Decolourization | | | |
|---|---|---|---|---|
| | Inorganic condition | | Organic condition | |
| | Control | Treatment | Control | Treatment |
| *Planococcus citreus* | .38 | .15(60.52%) | .45 | .12(73.33%) |
| *Bacillus brevis* | .38 | .20(47.36%) | .45 | .15(66.66%) |
| *Micrococcus luteus* | .38 | .15(60.52%) | .45 | .09(80.00%) |
| *Bacillus licheniformis* | .38 | .15(60.52%) | .45 | .13(71.11%) |

## CONCLUSION

From the present study it reveals that the four isolates can be successfully used for bio decolourization of triphenylmethane dye - Brilliant green. Shaking condition is preferable to static condition for incubation because more than 70% decolourization is found at shaking condition. The optimum incubation temperature is found to be $30^0$C. All of the organisms prefer neutral condition (pH 7.0) for decolourization. The rate of decolourization is found to decrease with increasing of pH. As a co-substrate glucose is more effective to generate redox equivalent as well as consequent dye decolourization by all of the organisms except *Bacillus brevis*. Organic supplement containing environment is more suitable to decolourize Brilliant green in compared to inorganic environment. From the basis of the result a suitable strategy can be developed to utilize such four organisms to decolourize Brilliant green contaminated effluent by maintaining suitable parameters of incubation before discharging into the river water.

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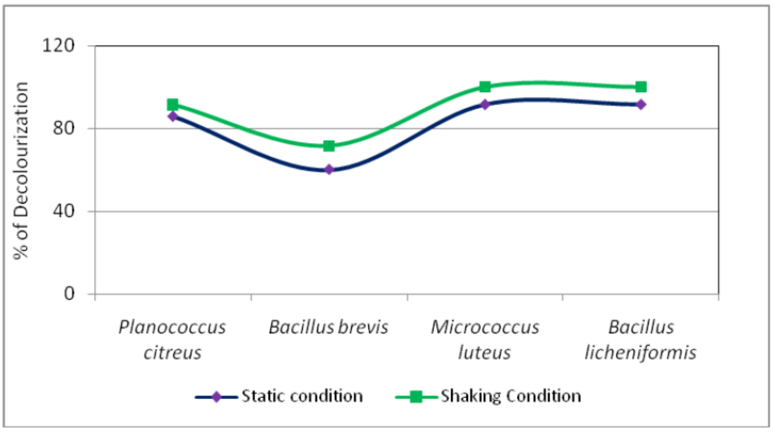



**Fig 1:** Effect of Brilliant green decolourization at static and shaking condition.

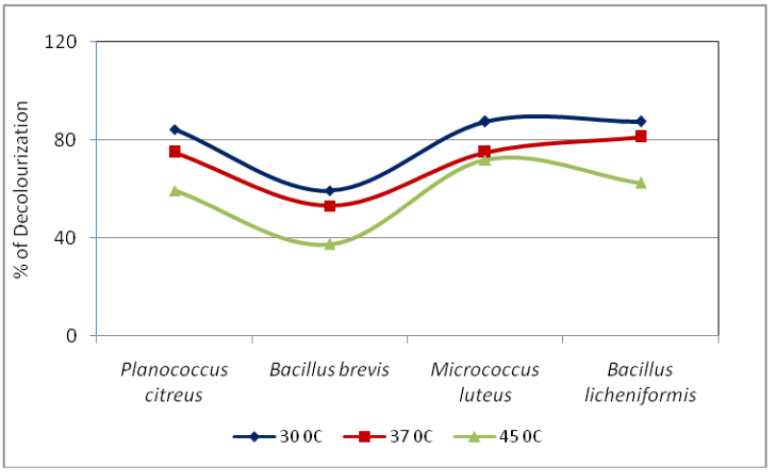



**Fig 2:** Effect of temperature on Brilliant green decolourization.

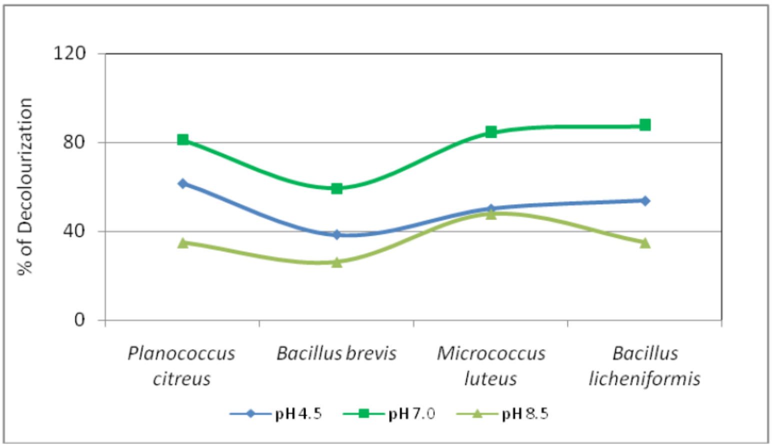
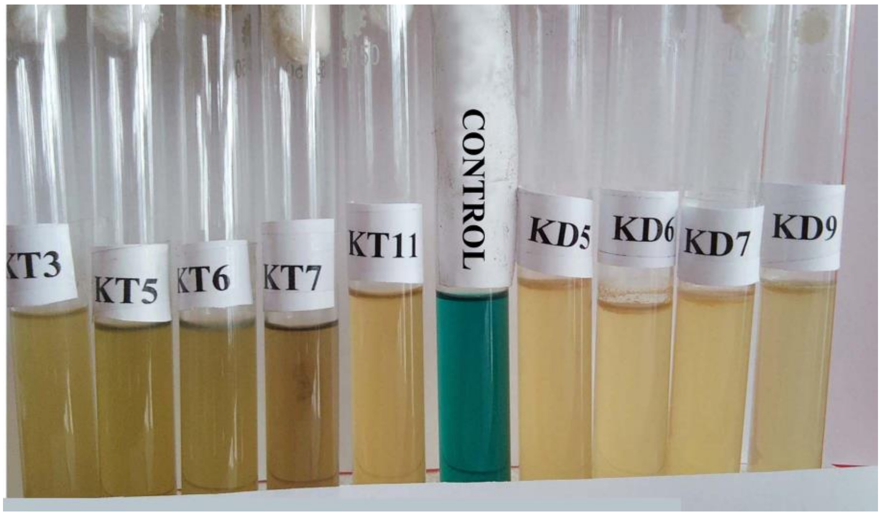



**Fig 3:** Effect of pH on Brilliant green decolourization.


KT3
KT5
KT6
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KT11
CONTROL
KD5
KD6
KD7
KD9