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BIODECOLORISATION REACTIVE ORANGE-16 BY *Klebsiella oxytoca* CMGS-3

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ABSTRACT

With the growing of world's population, and declined in the available water quantity and its resources. There is a need to search for a permanent solution to conserve and recycle the available water to recycle. For the treatment of waste water(effluents) many advanced techniques are developed like various physical, chemical methods are available, but they were having disadvantages in the proper detoxifying of dyes. These synthetic Reactive azo dyes are highly soluble in water, physicochemical process fails to remove dye completely from waste water. Presently, microbial degradation gave a promising results, in the degradation of dyes. Keeping these aspects, the present investigation was undertaken with an aim of isolation and characterization of potential azo reactive dyes degrading bacterial isolates from different environmental samples, out of those *Klebsiella oxytoca* CMGS-3 isolated from soil collected from surrounding soil of textile industry, showed maximum decolorisation of Reactive orange-16, with optimized abiotic and biotic factors like pH, temperature, salt concentrations, inoculums concentration, dye concentration, optimization of nutritional sources of energy etc , final decolorisation confirmed by UV-VIS Spectrophotometer.

Key Words : Reactive azo dyes, Effluents, Reactive orange-16, *Klebsiella oxytoca* CMGS-3, Waste water

INTRODUCTION

After Second world war increased human population leads to rapid industrialization, urbanization, green and white revolutions at the cost of natural resources and which also changed the life style of human. The conventional physical/chemical treatment methods cannot be completely remove azo dyes and their metabolites, generate significant amount of sludge that may cause secondary pollution problems and require complicated procedures[1]. Bioremediation is the microbial clean up approach is on the front line and priority research area in the environmental sciences. In this process microbes can acclimatize themselves to the toxic wastes and can transform various toxic chemicals to less harmful forms using the biotransformation enzymes system. In the current scenario, microbial or enzymatic treatment offers an indispensable, eco-friendly and cost-effective solution towards restoring azo dye polluted ecosystems. Also, the biological treatment system could help to reduce the enormous water consumption compared to conventional physicochemical methods[2]. The isolation of good dye decolorizing species requires screening and there isolated stains should have ability to degrade and detoxify textile dyes[3]. CMGS-3 isolated from soil collected from surrounding soil of textile industry, and identified as *Klebsiella oxytoca* CMGS-3 by biochemical and molecular techniques , showed a maximum decolorisation of Reactive orange -16, which is a widely used synthetic dye, posses a mutagenic property.

MATERIAL AND METHODS

Dye- Reactive orange-16 , purchased by sigma Aldrich, chemicals required for the decolorisation study purchased from Hi- media.
Decolorisation medium (DM)-Mineral salt media prepared as per with some modifications. MS medium was prepared by

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adding 10 mL of solution-2 to 100 mL of solution-1and adjusted pH-7.0. The solution-1 was prepared by adding gms/L of $Na_2HPO_4.2H_2O$ (12.00), KH_2PO_4 (2.00), NH_4NO_3 (0.50), $MgCl_2.6H_2O$ (0.10), $Ca(NO_3)_2.4H_2O$ (50.00 mg), $FeCl_2.4H_2O$ (7.50 mg) to 1000 mL distilled water. The solution-2 (trace element solution) was prepared by adding mg/L of $FeSO_4.7H_2O$ (0.10), $MnCl_2$. $4H_2O$ (3.0), $ZnSO_4.7H_2O$ (10.0), $CuSO_4.5H_2O$ (1.0), $MnSO_4.H_2O$ (0.017), $NiCl_2.6H_2O$ (2.0), $Na_2MoO_4.2H_2O$ (3.0), H_3BO_3 (30.0), $CuCl_2.2H_2O$ (1.0) into 1000 mL of distill water. MSM blended with 50mg/L reactive orange16 dye used as dye as sole source of energy for the isolates, un inoculated media serves as control for the decolorisation study.Isolation of isolate was done as per Anjaneya *et al.*[4]. Ten grams of soil sample or 10 mL of water sample (turbid) were added to 100 mL normal saline (0.9%) containing in 500 mL conical flasks containing 100 mL of normal saline and kept on rotary shaker at 120 rpm for one hour and left at room temperature without shaking until all suspended particles were settle down. The supernatant was used for the screening of RO-16 decolorizing microorganisms. Twenty ml of supernatant was inoculated to 100 mL Mineral Salt Medium (MSM) containing 50 mg/L RO-16 as sole source of carbon and incubated at 35^0 C till visible color changed in the flask . The flasks showing more than 50% reduction in the color intensity were selected and decolorisa-tion was confirmed by UV-Vis spectrophoto meter taking optical density at 540 nm. Again 20 mL of declorized culture was inoculated into fresh 100 mL DM (Decolorizing medium) contain ning flasks and were incubated once again and observed for the more than 50% of reduction in the initially added dye. Again the flasks showing maximum decolorization were selected for the isolation of RO-11 decolorizing microor-ganism. A 0.25 mL of culture from decolorized those flasks was taken out and inoculated on the MS agar medium containing 50 mg/L of RO-16 by pour/spread plate method. The plates were incubated at 35^0 C till visible growth appeared on the plates. The colonies showing clear zones around them were picked up and streak on the nutrient agar plates and study the cultural and morph ological characteristics. Further physiological and biochemical tests were performed to identify isolate up to genus level. The characterized cultures were subculture on MS agar containing 0.1% yeast extract and 100 mg/L RO-16 slants and after growth two slants were stored at 4 °C after adding 25% of sterile glycerol on the culture surface and one slant used for the further study.

Conformation of decolorisation- for the decolorisation study UV-VIS spectrophoto metric study was done ,the decolorized samples optical density checked at 500 nm corresponding to λ_{max} of the dye and With incr ease in incubation time the peak height at 500 nm goes on decreased and disappeared after 16 hrs of incubation indicates complete decolo risation of added RO-16 Dave and Dave[5].

RESULTS AND DISCUSSION

Reactive Orange-16 (RO-16) is brig ht yellow orange powder also called with other synonyms as Reactive Orange 3R, Reactive Orange KN-5R, Reactive Brilliant Orange KN-5R and it is a sulfonated non volatile, polycyclic aromatic compound.

Structurally it has one benzene ring with sulfonyl ethyl hydrogen sulfate attached to the naphthalene through azo bonding and naphthalene having one amino methyl, one OH and one sulfonate $NaSO_3$ groups makes it more recalcitrant in nature and also structurally it is called as mono azo reactive dye **(Fig.1)**. This is most widely used in cotton or viscose fiber dyeing and also used for cotton and viscose fiber and in printing. It is soluble in water and methanol.

Its color index number is C.I.17757, and CAS Registry Number: 12225,

Molecular Formula: $C_{20}H_{17}N_3Na_2O_{11}S_3$,

Molecular Weight: 617.54, λ_{max} of 500 nm.

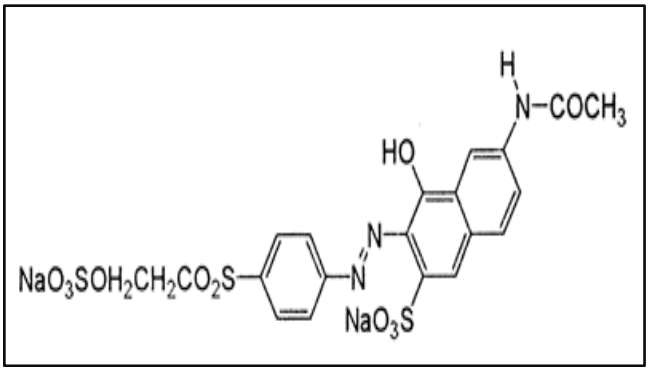

Fig. 1: Structure of Reactive Orange-16.

Optimization various parameters for maximum decolorisation of RO-16

Effect of static and shaking (aeration) conditions

Isolate CMGS-3 showed 94.1% of decolorization within 16 hrs under static condition. In contrast under shaking condition these organisms showed only, 62.4% of RO-16 decolorization respectively. Therefore, further optimization studies were performed only under static condition **(Fig. 2)**.

Optimization of temperature

The decolorization of RO-16 was tested over wide range temperatures from 20 to 50° C with an interval of 5° C. CMGS-3 showed maximum decolorisation of 95% at 35° C considered as optimum temperature for the maximum decolorisation RO-16**(Fig. 3).**

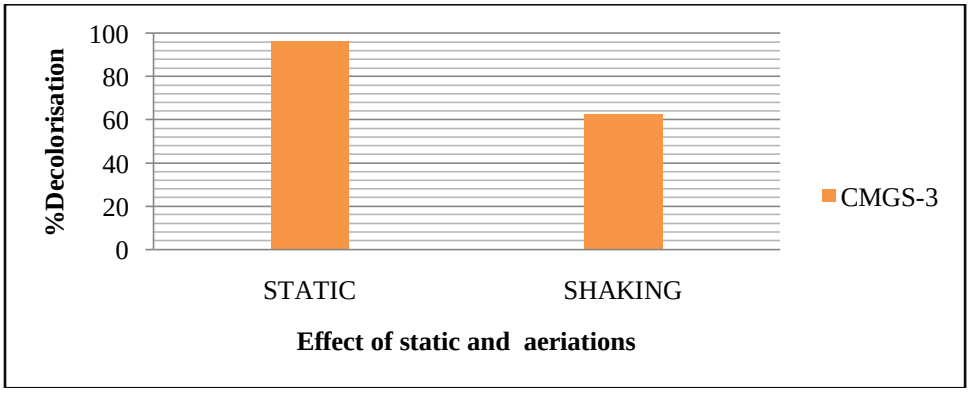

Fig. 2 : Effect of static and shaking condition on the decolorisation of RO-16 by isolate CMGS-3

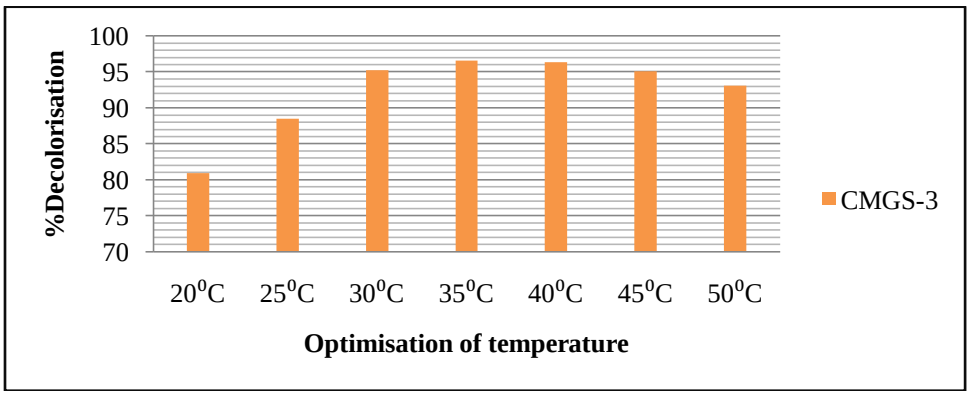

Fig. 3 : Optimisation of temperature condition on the decolorisation of RO-16 by isolate CMGS-3

Optimization of pH

The optimization of pH for maximum decolorization of RO-16 by isolate was determined using over a wide range of pH from 4 to 14 with the interval of 1. CMGS-3 showed maximum decolorisation of 95% and 96.3% at pH-9 respectively **(Fig. 4).**

Optimization of inoculum size

The optimization of inoculum size for RO-16 decolorization by isolate was determined with different volumes of initial bacterial inoculums (bacterial concentration was 10^6/mL) from 5 ml to 20ml/L voloume/volume . Isolate CMGS-3 showed more than 96% of decolorization at 10% of inoculum size under static incubation with optimum conditions with pH-8 and temperature 35° C **(Fig. 5).**

Optimum incubation time for maximum RO-16 decolorization by isolate CMGS-3 under optimum condition

To know the minimum incubation time required for the maximum decolorisation of RO-16 by each isolate by incubating isolate separately in 100 mL of DM with different concentrations of RO-16 (100 to 1000 mg/L) under static conditions with optimized parameters.

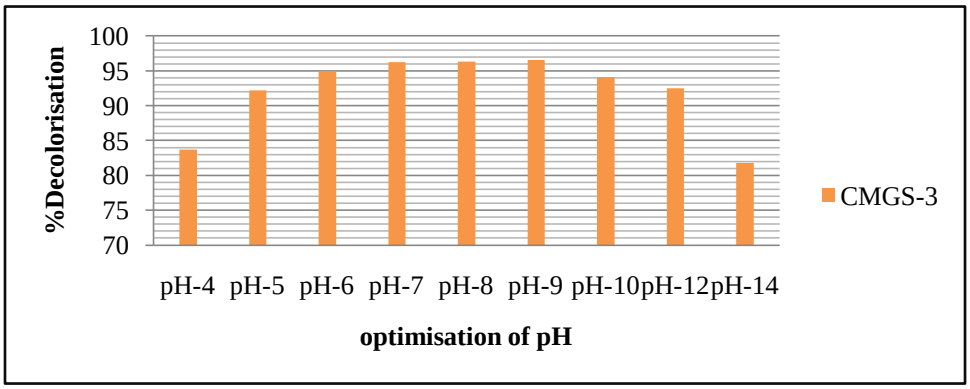

Fig.4 : Optimisation of pH condition on the decolorisation of RO-16 by isolate CMGS-3.

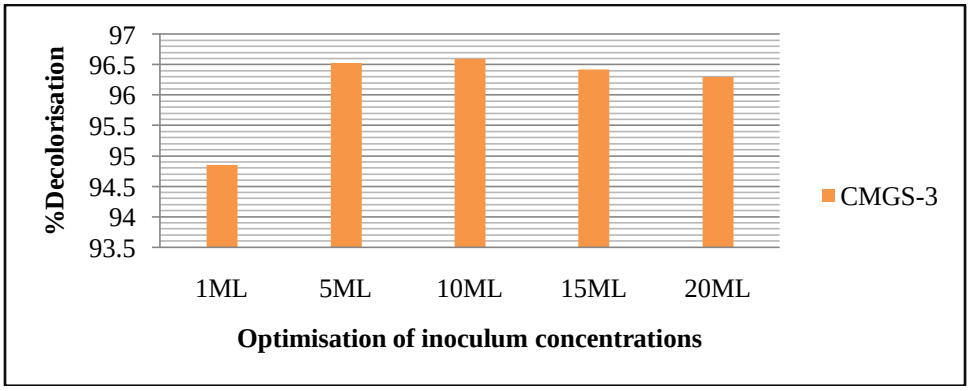

Fig. 5 : Optimisation of inoculum concentrations on the decolorisation of RO-16 by isolate CMGS-3

CMGS-3 showed 96% of decolonization of RO-16 up to 200 mg/L within 16 hrs of incubation. Further increased in the dye concentration decreasing of decolorisation was noticed **(Fig. 6)**.

Effect of salt concentration

Effect of salt concentration on the RO-16 decolorization efficiency of isolate CMGS-3 was checked by incubating DM with different concentration of NaCl (1 to 5%) . It was observed that CMGS-3 isolate was not tolerated more than 2% of NaCl, however isolate was able to decolorized around 70% of initially added RO-16 at 5% salt concentration and showed maximum decolorisation at 1% of salt concentration.**(Fig.7).**

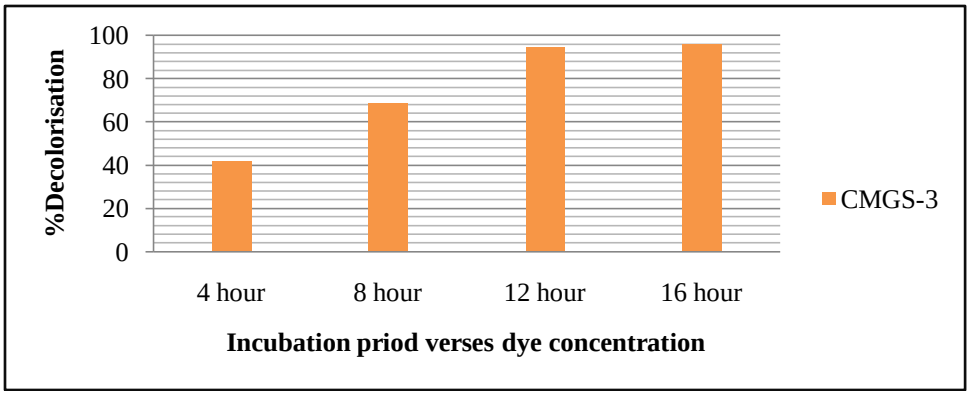

Fig. 6 : Effect of dye concentrations on the decolorisation of RO-16 by isolate CMGS-3

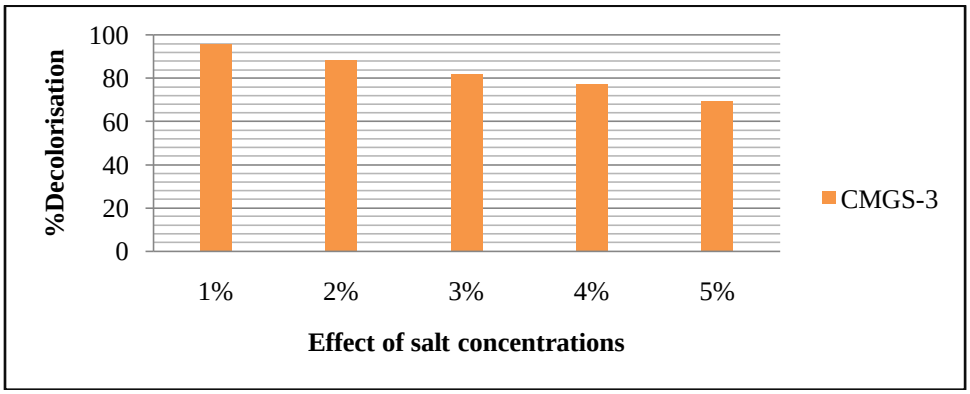

Fig. 7 : Effect of dye concentrations on the decolorisation of RO-16 by isolate CMGS-3

Effect of additional nutritional sources

Additional nutritional source maximizes the decolorization capacity of the organism was performed with various carbon and nitrogen sources. Carbon sources selected like glucose, sucrose and starch were not shown any additional increase in the decolorization activity of three bacterial isolate tested. Among nitrogen sources selected only yeast extract alone showed increase in the RO-16 decolorization efficiency of isolate by reducing the incubation time from 72 hrs to 16 hrs. CMGS-3 a showed 96.53% decolorization within 16 hrs of incubation. However, ammonium nitrate and potassium nitrate showed reduction in the decolorization capacity of the isolate.

Optimization of Yeast extract concentration for maximum decolorization RO-16.

Further to determine the optimum concentration of yeast extract for maximum decolorization of RO-16 by different isolate, the DM was incubated with different concentrations of yeast extract ranges from 0.05% to 0.2%. The results showed that maximum decolorization was with 0.1% of yeast extract for the isolate, further increased in concentration of yeast extract was not shown any effect on the efficiency of RO-16 decolorization .

UV-VIS Spectroscopy

UV-Vis scan (200-900 nm) of spent medium of before and after RO-16 decolorized by CMGS-3 showed single well defined peak at 500 nm corresponding to λ_{max} of the dye. And no peak was observed at 500 nm in complete decolorized spent medium taken after 16 hrs of incubation .This clearly indicates complete decolorization of RO-16 by CMGS-3.

The excessive discharge of the effluents from the textile industries contains toxic chemicals such as azo reactive dyes which adversely affect the natural resources, soil fertility and aquatic organisms and disturb the integrity of the ecosystem [6].

Due to heavy discharge of synthetic dyes leads accumulations of toxics which effects on aquatic life and humans, due to presence of

aromatic compounds, makes dyes to xenobiotic, carcinogenic and mutagenic[7,8]. In India 70% of water is polluted, which impact the life of aquatic creatures due to the change in the water quality[9]. In textile industries 93% of the outgoing water comes out as colored water and contains high concentration of organic compounds and heavy metals[10].

Indian Government has proposed laws by considering the adverse effect on environment and health of people due to the effluents being thrown out of the textile and dye industries. Many laws were introduced by Government of India to control pollutions of water bodies and inferred through the Central Pollution Control Board (CPCB) and to maintain the minimum acceptable standards. Imposition of strict prevention and central pollution act in 1974, many industries started building up of effluent treatment plants to treat the effluents before discharge in to natural water bodies. Most of methods employed are of physical, chemical types are not meet the standard preferred by CPCB and having disadvantages in the proper detoxifying of dyes[11]. With the aid biological methods, that is with the use of microorganisms can achieve complete mineralization of synthetic aromatic dyes. We adapted various methodologies to optimize the environmental, nutritional and operational parameters to achieve optimum decolorisation Reactive Orange-16 (RO-16) by selected CMGS-3 strain, which was isolated from textile treatment unit and surrounding soil of textile industry identified as *Klebsiella oxytoca* CMGS-3 by biochemical and molecular methods. Among the abiotic factors like pH and temperature are play vital role in the determination of azo reactive dyes decolorization efficiency of newly isolated bacterial strains. As pH and temperature directly effect on the physiology and growth of bacteria which in term affects the efficiency of dye degradation. CMGS-3 showed maximum decolorisation of 95% and 96.3% at pH-8 ,However, our results are revealed that isolate have a capacity to decolorize more than 70% at pH-5 though pH-12 and maximum at pH 8 and 9. studied[1] Reactive violet-5 also degraded in the wide range of pH 6 to pH9 with an optimal of pH-7.5 by *Paracoccus* sp. GSM-2[12], where *Micrococcus glutamicus* in combination with *Proteus vulgans* completely decolorized the Scarlet-R under static condition in acidic range of pH 5 to pH 8 with optimum pH 7.0[13]. With temperature isolate CMGS-3 showed maximum decolorisation a of 95% at 35° C considered as optimum temperature for the maximum decolorisation RO-16 reveals that isolate is mesophilic in nature, similarly with our results Decolorization of Acid Dyes by *B. cereus* and *P. aeruginosa* 37^0C was found to be optimum[14]. Singh *et al.* have reported[15] for the maximum decolorisation 92.38% of Acid orange by *Staphylococcus hominis* RMLRT03 strain was found at 35°C. Many reports[16,17] revealed that most of azo reactive dyes are decolorized by the various microorganisms in the range of 30 to 45℃. Our results in accordance with earlier reports.

For effect of dye concentration were studied, isolate CMGS-3 performed maximum decolori -sation up to 200 mg/L of RO-16 with 16 hrs of incubation further increase in the incubation time does not showed effective decolorisation. Again as dye concentration increases the percent of decolorisation was reduced. This may be due to presence of substituent groups like sulfamine groups (SO_3H) and Cl groups may act as detergents and surfactants which easily inhibit the bacterial growth. It is well known phenomenon or fact that higher concentration of reactive azo dyes inhibits the nucleic acid synthesis and cell growth also. Therefore, the effect of dye concentration on the growth and decolorizing efficiency of the organisms is an important consideration for use as candidate in the field application, Pearce *et al.*[18] has been proposed that efficiency of microbial decolorization through a combination of factors including the toxicity imposed by dye at higher Concentration. Inoculum concentration varies from species to species isolate CMGS-3 showed maximum decolorisation at 10ml/L of initially added inoculums concentration, in the report of Ponraj *et al.*[19] have reported that decolorization activity of *Bacillus* sp. has high (86.72%) only at 4% of inoculum concentration , further Mathew and Madamwar[20] reviewed that for increasing decolorisation percent of inoculum concentration not plays more significant role. Isolate

CMGS-3 showed maximum decolorisation up to 2% salt concentration similarly with our reports Oturkar *et al.*[21] showed *Bacillus lentus* BI377 could tolerate up to 2% salt concentration without change in the percent decolorisation and time period. Carbon and nitrogen sources provide energy for the growth and survival of the bacteria and also transfer reducing equivalent to the dyes for azo bond cleavage[22] but in our reports there was no change with the addition of carbon source reveals isolate uses dye as a sole source of carbon , with nitrogen sources only yeast extract gave a good results and 0.1% yeast extract considered as better source and volume in the decolorisation by isolate like our reports Our results are accordance with recent reports of Rajeshwari *et al.*[23] where the bacterial isolate *Lysinibacillus* RSV-1 showed maximum 95% of decolorization with 100 mg/L initially added dye in the presence of yeast extract and not with addition of other inorganic sources and also with urea. They also concluded that yeast extract act as dual source of carbon and nitrogen in decolorization of various synthetic azo dyes for many bacterial isolates. Finally complete decolorisation studied by UV-VIS Spectro phometer lowering optical density at 500nm at 16 hours.[24-28]

CONCLUSION

The main objectives of the present study were investigating the bacterial degradation of aromatic hydrocarbon derived Reactive dyes, which taken leading position in the production of Reactive dyes. Based on the results of present investigation, we suggest and propose utilization of *Klebsiella oxytoca* CMGS-3 for field application in the treatment of dyeing industry effluents especially effluents containing reactive dyes.

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