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DECOLORISATION OF REACTIVE ORANGE-16 BY LOCAL ISOLATED STRAIN *Enterococcus casseliflavus* CMGS-1 STRAIN

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ABSTRACT

The enhancement of the usage of synthetic colors and large quantity of waste was generated. The use of synthetic materials goes on increased day by day since from 1970's which leads to pollute the mother planet in all sides. As the water is the main natural resource, which is drastically utilized in all fields especially more in textile, tannery, paper and food processing industries. Most of these industries and the urbanization established on the banks of rivers and streams and which contributes the pollution of surface and ground water heavily so water becomes precious and we all require a good quality of water for healthy life on the earth, to solve these problems a strong biological aid is needed, in this direction *Enterococcus casseliflavus* CMGS-1 strain isolated from soil sample collected from textile effluent treatment unit and a Reactive orange-16(RO-16), a synthetic dye which is widely used in textile sector, isolate showed a complete decolorisation RO-16 with optimized parameters for the maximum decolorisation of the dye studied by UV-VIS Spectrophotometer.

Key Words : Reactive orange-16(RO-16), *Enterococcus casseliflavus,* Textile effluent treatment, Natural resource, Dye

INTRODUCTION

Earlier days natural colorants were used which were non toxic and eco-friendly and are prepared from plant sources like Roots, berries, bark, leaves, wood and lichens etc. Researcher reviewed that natural dyes were practiced in China dated 2600 years ago, in 1500BC Mohenjo-Daro and Harappa various natural colors were used to coloring the clothes and also in bible it is mentioned use of Saffron during 2500 BC.

The best example for the natural color is turmeric, a naturally occurring yellow color dye, it is powerful antiseptic and indigo another natural dye gives cooling effect[1]. When these natural dyes were replaced by the synthetic dyes, the actual problem of water pollution was started. The discovery of synthetic dye Mauveine by Perkins in 1856 has provided a tool for the production of wide range of dyes that are color fast and come in a wider color range and brighter shades.

These synthetic dyes were widely used in the textile, leather, pharmaceuticals, and food industries and are manufactured from the compounds obtained by the distillation of coal tar, majorly dyes consists intermediates like Benzene (C_6H_6), Toluene ($C_6H_5CH_3$), Naphthalene ($C_{10}H_{30}$), Anthracene ($C_{14}H_{10}$), Phenol (C_6H_5OH), Cresol (C_7H_7OH), Acridine ($C_{13}H_9N$), and Quinoline (C_9H_7N). These intermediates are hydrocarbons in which one or more of the hydrogen atoms are replaced by groups such as the nitro group ($-NO_2$), amino group ($-NH_2$), hydroxyl group (-OH), sulfonic acid group ($-OSO_3H$), and others. Examples of such compounds are nitrobenzene ($C_6H_5{\cdot}NO_2$), aniline ($C_6H_5{\cdot}NH_2$), β-naphthol ($C_{10}H_7{\cdot}OH$), and β-naphthalene sulfonic acid ($C_{10}H_7{\cdot}SO_3H$). Many of these dyes are polycyclic aromatic hydrocarbon compounds as their primary structures and makes more recalcitrant in nature.

Dye consists of two groups one is chromophores and another auxochromes, combining of these becomes a synthetic dye.

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Chromophores are a group, responsible for producing a color. Some important chromospheres are N=O, $-NO_2$, -N=N-, -C=O, C=S, -C=N, and $(CH\text{-}CH)_n$, and the compounds bearing chromophores are known as chromogens.

Auxochromes are compounds helps to dye get attached to the fiber by means of stable chemical bonds. These chemical bonds are formed by groups that are either acidic or basic in nature. Examples are -OH, -COOH, $-SO_3H$ (all acidic) and $-NH_2$, -NHR, $-NR_2$.

Reactive azo dyes are extensively used in the textile industries and are classified by its chemical structure of chromophore dyes are divided into Azo (monoazo, diazo, triazo, polyazo), anthraquinone, phthalocyanine and triaryl methane dyes are the most important groups. Among the synthetic dyes azo dyes produce clear and strong colors and are available in various shades and cost effective . They are primarily used in cotton, leather, cosmetics and food.

It is estimated that approximately dye discharged by textile processing, primarily belongs around class of Reactive Azo dyes (36%), Acid (25%), Direct (15%) dyes and followed by remaining other classes of dyes[2]. Among the different types of dyes reactive azo dyes were widely used. Most of the azo dyes are water soluble and readily to absorb through skin contact and inhalation leading to the risk of cancer and allergic reactions, an irritant for the eyes and highly toxic, if inhaled or consumed (Nikulina *et al.*, 1995). 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[3]. Physical and biological treatment does not remove completely synthetic dye s water resources, nowadays biological treatment taking a leading role in declorisation section , that is use of microorganisms we isolated a bacterial strain *Enterococcus casseliflavus* CMGS-1 strain, from soil sample collected from textile effluent treatment unit, performed a maximum decolorisation of reactive orange -16 which is useful isolate in future with treatment of other reactive dyes.

MATERIAL AND METHODS

Dye- reactive orange-16 purchased by sigma Aldrich details of dye is given below- Reactive Orange-16 (RO-16) is bright 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 and chemicals required for the decolorisation study purchased from Hi- media.

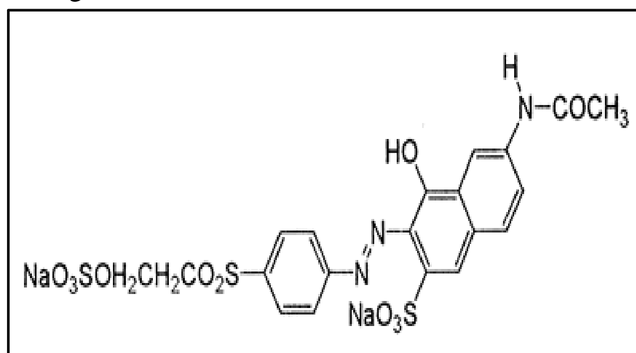

Fig. 1: Structure of reactive orange-16

Media for the decolorisation- mineral salt media prepared as per[4] with some modifications. MS medium was prepared by 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.*[5] 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 decolorisation was confirmed by UV-Vis spectrophotometer taking optical density at 540 nm. Again 20 mL of declorized culture was inoculated into fresh 100 mL DM (Decolorizing medium) containing 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-16 decolorizing microorganism. 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 morphological 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 ^oC after adding 25% of sterile glycerol on the culture surface and one slant used for the further study.

Conformation of decolorisation : it is confirmed through UV-VIS spectro photometric study,the decolorized sample optical density checked at 500 nm corresponding to λ_{max} of the dye and . With increase in incubation time the peak height at 500 nm goes on decreased and disappeared after 16 hrs of incubation indicates complete decolorisation of added RO-16 Dave and Dave.[6]

RESULTS AND DISCUSSION

Optimization of various biotic and abiotic parameters

Study of effect of static and shaking (aeration) conditions

The results showed that decolorization of RO-16 by CMGS-1 was maximum of 95.1% within 12 hrs under static condition. In contrast under shaking condition showed only, 63.8%, decolorization respectively **(Fig. 2)**. Isolate CMGS-1 performed maximum decolorisation in static condition , it revealed isolate needs a lesser oxygen for the decolorisation, similarly in some article they reviewed the decolorization of azo dyes was maximum in strictly anaerobic conditions, although it also occurred under semi-anaerobic ones[7]. Similar results were observed in studies on pure bacterial strains such as *Pseudomonas luteola, Proteus mirabilis, Pseudomonas sp. SUK1, Micrococcus glutamicus NCIM-2168*.[8-12] Under anaerobic conditions enzyme activity was high, only small amount of oxygen needed.

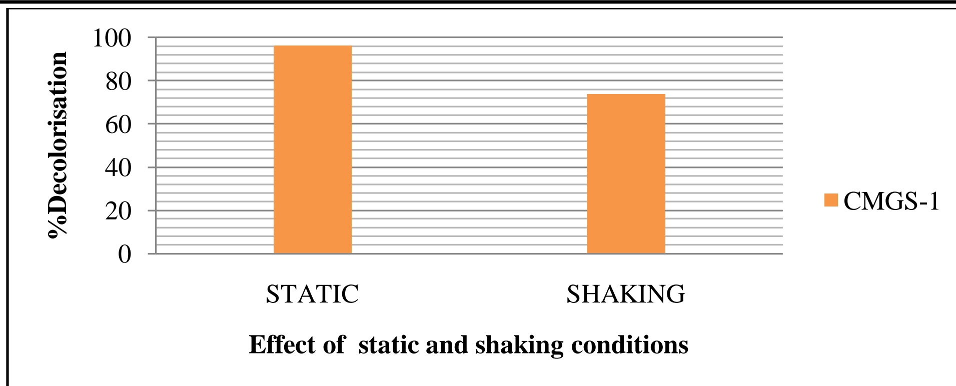

Fig. 2 : Study of static and shaking condition on decolorisation of RO-16 by isolate CMGS-1

Optimization of temperature for maximum decolorisation

The decolorization of RO-16 was tested over wide range temperatures from 20 to 50° C with an interval of 5° C. It was observed that isolate CMGS-1 showed maximum decolorisation at 40° C of 96% and at 50° C it was 60% of decolorisation **(Fig. 3)**. Our isolate showed thermo stable activity it has been shown that with certain whole bacterial cell preparations the azoreductase is a stable enzyme it is relatively thermostable and can remain active up to temperatures of 60^0c over short periods of time[13] and for the dye degradation stable azoreductase enzyme is very important.

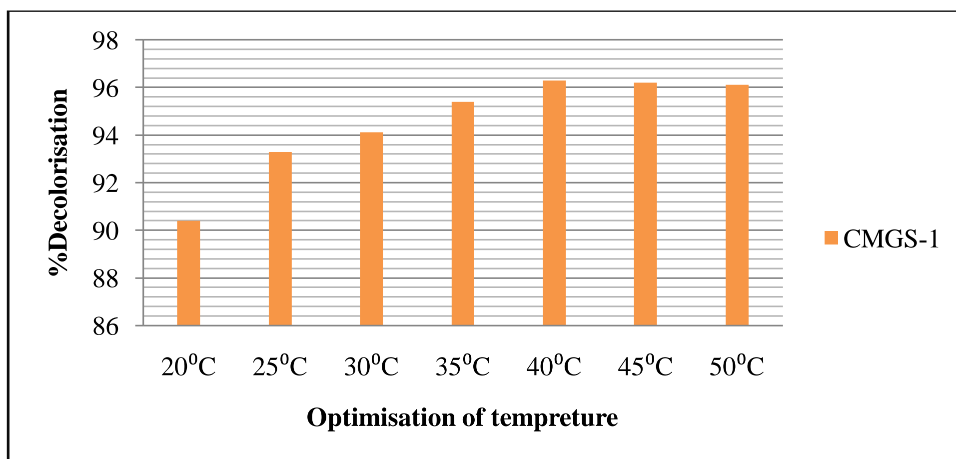

Fig. 3 : Study of temperature condition on decolorisation of RO-16 by isolate CMGS-1

Optimization of pH for maximum decolorisation

The optimization of pH for maximum decolorization of RO-16 by three isolates was determined using over a wide range of pH from 4 to 14 with the interval of 1. Isolate showed maximum of around 90% of RO-16 decolorization at pH-7 to 9. Further isolate CMGS-1 showed consistent decolorisation above 50% in all ranges of pH tested and showed maximum decolorization of 96.4% at pH-9 and was optimum pH for the isolate to decolorize maximum amount of dye **(Fig. 4)**. revealed Bacterial cultures generally exhibit maximum decolorization at pH values near 7-8. Bacillus species exhibited decolorization activity in the range of pH 5-8. At pH 3 and 4 decolorization observed was 15 and 38% respectively. The isolate showed more or less constant decolorization from pH 5 to 8. Maximum (98%) being at pH 8. The isolate exhibited decolorization ability in the range of pH 5-8. Our report is accordance with previous reports; *Pseudomons aerugi nosa* BCH exhibited best decolorization at pH 8 as stated by Shekhar et al[14].

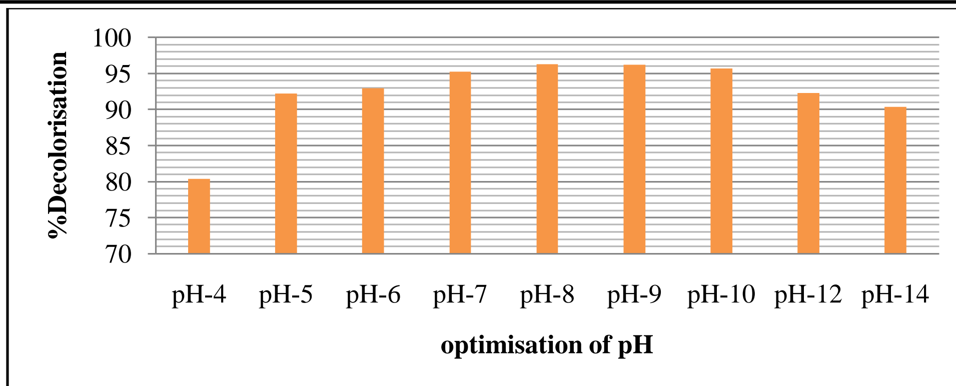

Fig.4 : Study of pH conditions on decolorisation of RO-16 by isolate CMGS-1.

Optimization of inoculum concentration

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). CMGS-1 showed more than 96% of decolorization at 5% of inoculum size under static incubation with optimum conditions with pH-8 and temperature 40° C **(Fig. 5).** Inoculum concentration varies from one organisms to another, Sudha and Balagurunathan, studied decolorization activity of *Bacillus licheniformis* specially in reactive azo dyes 20% of inoculum size require for the maximum decolorisation.

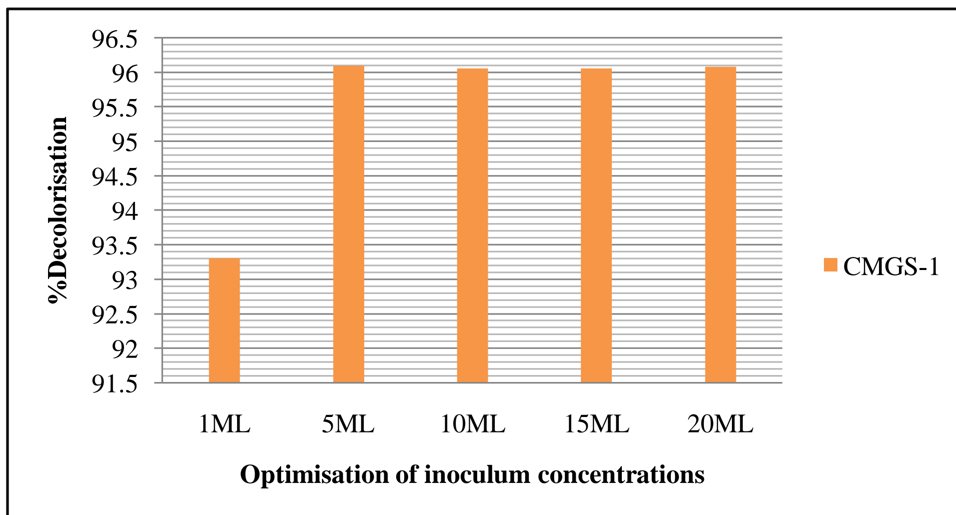

Fig. 5 : Study of inoculums concentrations on decolorisation of RO-16 by isolate CMGS-1

Decolorisation study of maximum RO-16 decolorization by isolates under optimum condition.

To know the minimum incubation time required for the maximum decolorisation of RO-16 by each isolates by incubating each isolate separately in 100 mL of DM with different concentrations of RO-16 (100 to 1000 mg/L) under static conditions with optimized parameters. Results showed that all three isolates were decolorized the initially added RO-16 maximum of more than 95% within 16 hrs of incubation. CMGS-1 showed maximum decolorization of 96.53% with 400 mg/L within 12 hrs, Further increased in the dye concentration decreasing of decolorisation was noticed in isolate percent decolorisation of RO-16 with dye concentrations above 500 mg to 1000 mg/L was observed up to 24 hrs of incubation with each isolate. **(Fig 6)** with our report Cetin and Donmez[15] studied that higher the dye concentration requires longer the time duration and Wang *et al.* have reported that the decolorization rate of Reactive Black 5 by *Enterobacter* sp. EC3 was decreased with increase in initial dye concentration.

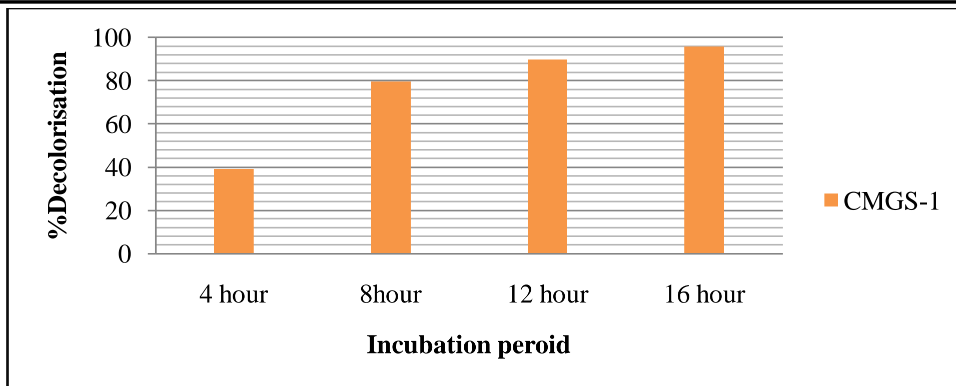

Fig. 6 : Effect of the dye concentration on decolorisation of RO-16 by isolate CMGS-1

Effect of salt concentration in decolorisation study

Effect of salt concentration on the RO-16 decolorization efficiency of isolate was checked by incubating DM with different concentration of NaCl (1 to 5%) and results are shown **Fig. 7**. It was observed that CMGS-1 not tolerated more than 2% of NaCl, however isolate was able to decolorized around 70% of initially added RO-16 at 5% salt concentration similar with results, Bheemaraddi *et al.*[16] have reported *Paracoccus* sp. shown decolorisation up to 6% salt concentration. Khalid *et al.*[17] reported salt tolerant bacterium may facilitate the development of biological treatment azo dye for the bioreactor because waste water from dyestuff manufacturing and textile processing industries shows presence of various acids, alkalis, metal ions and salt as impurities.

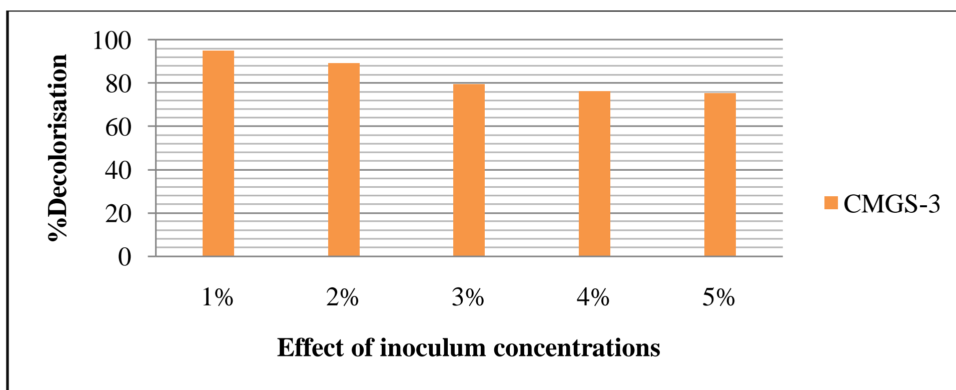

Fig. 7 : Study of salt concentrations on decolorisation of RO-16 by isolate CMGS-1

Effect of additional nutritional sources

To check the effect of additional nutritional source on 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 all three bacterial isolates 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. Isolate CMGS-1 showed 96.23% of decolorization within 12 hrs of incubation, However, ammonium nitrate and potassium nitrate showed reduction in the decolorization capacity of the isolate, Carbon source like glucose, sucrose and starch were used none of the isolate had shown any additional effect on decolorization efficiency in terms of either increase in percent decolorization or in reduction decolorisation time. These revels that microbe's uses dye as a sole source of carbon. On other hand there is a decrease in decolorization percent after addition of some carbon sources. It is reported

that some of the sugars may inhibit the decolorization of azo dyes because it's effect as catabolite repression[9] and isolate CMGS-1 showed maximum decolorisation in only yeast extract as compare to other nitrogen sources.

Optimization of yeast extract concentration for maximum decolorization RO-16

Further to determine the optimum concentrate-ion of yeast extract for maximum decolorize-tion of RO-16 by different isolates, 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 with our reports *Paracoccus* sp. by Bheemaraddi *et al.*[16] and Hu[18] reported that decolorisation efficiency of *Psedomonas luteola* was directly related to the concentration of yeast extract.

UV-VIS Spectrometric study

UV-Vis scan (200-900 nm) of spent medium of before and after RO-16 decolorized by isolate CMGS-2 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 12 hrs of incubation. This clearly indicates complete decolorization of RO-16 by CMGS-1, similarly the decolorisation studied by various researchers were Similarly many reports have been reported that Reactive red - 195 at 542 nm by *Micrococcus glutamicus* by Sahasrabudhe *et al.*[19-21]. Metanil yellow at 430 nm by *Lysinibacillus* sp. AK2 (Anjeneya *et al.,* 2011). Reactive red-2 at 538 nm by *Paracoocus* sp., sulfonated toxic azo dye Direct red-81 at 511 nm by *Enterococcus fecalis* YZ66.

CONCLUSION

Environment pollution arising everywhere , specially reactive dyes which were the main sources of contaminations , reactive dyes are made up of polycyclic aromatic hydrocarbons which cannot be degradable easily, because of their complex structure and chemical compositions creates a harmful effect to the nature and its living beings, physical and chemical methods fails in the proper treatment of synthetic , nowadays microorganisms are the tools, specially bacterial strain , these organisms completely mineralize dye converts nontoxic, in the direction *Enterococcus casseliflavus* CMGS-1 showed a maximum decolorisation of Reactive orange-16, the strain can be useful for the treatment of textile effluents.

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