International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 6, Issue 5 -2019

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2019.06.05.014

"Decolorisation of Reactive Red-120 Azo Dye by *Micrococcus* Sps"

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Abstract

The study was conducted in the field of biodecolorisation with commercially available textile dye that is Reactive red -120. For Decolorisation study a novel bacterial stain, which tentatively identified as *Micrococcus Sps* strain, based on morphological, cultural characteristics and biochemical tests was isolated from the dye contaminated soil from MIDC waste water treatment unit Sholapur Maharashtra. The bacterial decolorization efficiency was highly dependent on varies biotic and abiotic factors such as Static, Shaking, pH, Temperature, Inoculum size, carbon source Nitrogen source, salt concentration to enhance the dye decolorization efficiency of strain by optimizing various biotic and abiotic factors for each bacterial isolate and 0.1% of yeast extract enhance the decolorization efficiency of these bacterial isolate significantly under static conditions compared to shaking condition.

Keywords: *Micrococcus Sps*, Static, Shaking, pH, Temperature, decolorization.

Introduction

One of the major problems that humans are facing is the restoration of the contaminated environment. Textile dye contributes as the most important environment polluting agents, textile industries are the largest consumers of dyes and pigments accounting 80% of total production (Thakur, *et al*., 2014). Synthetic dyes are complex aromatic molecular structures which are intended to be stable and consequently are difficult to degrade. At present, there are more than 100,000 dyes available commercially (of which azo dyes, represent about 70% on weight basis), and over -1 million tons dyes are produced per year, of which 50% are textile dyes. In India alone, dye stuff industry produces around 60,000 metric tons of dyes, which is approximately 6.6% of total colorants used worldwide. The largest consumer of the dyes is the textile industry accounting for two third of the total production of dyes. The dyes intended to be used for a specific application must conform to the quality criteria set for the commercial acceptability of the end product (Puvaneswari *et al.,* 2006).Synthetic dyes are widely used in many fields of advanced technology, e.g., in various kinds of the textile, paper, leather tanning, food processing, plastics, cosmetics, rubber, printing and dye manufacturing industries. Synthetic dyes are also employed in ground water tracing, for the determination of specific surface area of activated sludge, sewage and wastewater treatment, etc (Velarde, 2014).

Environmental pollution has recently become a severe problem worldwide, Dyes are one of the most hazardous chemical compound classes found in industrial effluents and need to be treated since their presence in water bodies reduces light penetration, precluding the photosynthesis of aqueous flora they are also aesthetically objectionable for drinking and other purposes and can cause allergy, dermatitis, skin irritation and also provoke cancer and mutation in humans (Ogugbue *et al.*, 2012).

Mills discharge millions of gallons of this effluent as hazardous toxic waste, full of color and organic chemicals from dyeing and finishing salts. Presence of sulphur, naphthol, vat dyes, nitrates, acetic acid, soaps, chromium com- pounds and heavy metals like copper, arsenic, lead, cadmium, mercury, nickel, and cobalt and certain auxiliary chemicals all collectively make the effluent highly toxic (Rita, *et al.*, 2012).

More than 10,000 dyes are used in the textile industry and 2,80,000 tons of textile dyes are discharged every year worldwide as untreated effluents in the form of wastewater into public drains that eventually empty into rivers (Hsueh *et al.*, 2005).

Azo and nitro compounds have been reported to be reduced in sediments of aquatic bodies giving rise to potentially carcinogenic amines(Chen, 2006).

Bioremediation is the microbial clean up approach is on the front line and priority research area in the environmental sciences. This field has recent origin and grown exponentially over the last two decades. The microbial decolorization and degradation of azo dyes has been of considerable interest since it is inexpensive, eco-friendly, and produces a less amount of sludge (Kalyani *et al*., 2008). The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. Wide range of microorganisms including bacteria, fungi, yeasts, actinomycetes and algae capable of degrading azo dyes have been reported (Chen *et al*., 2003; Daneshwar *et al*., 2007). Most studies on azo dye biodegradation have focused on bacteria and fungi in which various fungal cultures mainly belonging to white rot fungi has been used to develop bioprocesses for mineralization of azo dyes(Parshetti *et al*., 2007).

Microbial communities are of primary importance in degradation of dye contaminated soils and water as microorganisms alter to dye chemistry and mobility through reduction, accumulation, mobilization and immobilization (Kumar *et al*., 2012). In recent years, biodegradation has become a viable alternative and proven to be a promising technology. Microorganisms have been successfully employed as sources for bioremediation (Khan and Husain 2007). Bioremediation is gaining its significance in utilizing the biological activity of microorganisms to degrade toxic chemicals in the environment (King *et al*., 1998). The bacterial degradation of azo dye is usually nonspecific and bacterial decolorization is normally faster Bacteria Halophiles have been reported to be involved in the dye decolorization (Salahuddin *et al*., 2007).Bacterial species also used in the degradation process.

Materials and Methods

Information of dye-Reactive Red 120 procured from the colorise dye Ahmadabad (Gujarat).It is a polycyclic aromatic hydrocarbon compound, which cannot degrade easily for this reason Reactive Red - 120 dye is used . Dye information- Name: - Reactive Red 120 (RR-120),Molecular formula- C44 H24 Cl2 N14 Na_6, O_{20} S_6, Molecular weight: - 1470 gm/mol, Water solubility: - 70 (gl^{-1}), ƛmax: - 520,Class:-Diazo (-N≡N- bond)**,**CAS Registry no: - 61951-82-4**,** Molecular structure: - Double azo class dye.

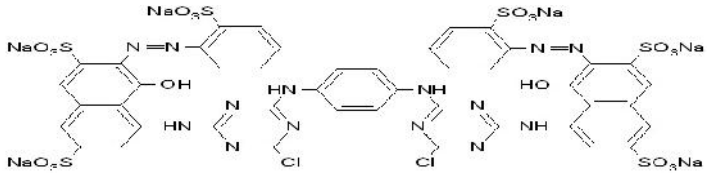

Figure- Structure of Reactive Red 120

Chemicals-The chemicals, reagents, media and other requirements used in the study were of analytical grade and procured from Hi-media pvt- ltd Mumbai.

Collection of sample-The soil and water samples were collected from MIDC area of Sholapur city of Maharashtra, India. The samples include particularly from a) soil of sewage treatment plant. b) Dye contaminated Soil. c) Effluents of inlet and outlet line (dye contaminated water).

All the samples were collected in sterile screw cap bottles and brought to the laboratory used immediately or they were stored in the refrigerator (4◦C) if delayed.

METHODS: Mineral salt medium for the decolorisation (Brilon *et al*., 1981) with little modifications.

$Na_2HPO_4.2H_2O$ -12.00g ,KH_2PO_4 -2.00g,NH_4NO_3 -0.50g, $MgCl_2.6H_2O$ -0.10g, $Ca(NO_3)_2.4H_2O$ -50.00mg, $FeCl_2.4H_2O$ - 7.50mg , Distilled water -1000.00ml, pH -7.00.

Trace elements solution (mg/l)-$FeSO_4.7H_2O$ -0.10, $ZnSO_4.7H_2O$- 0.05, $CuSO_4.5H_2O$- 0.02, $CaCl_2.6H_2O$ - 0.005, $MnSO_4.H_2O$ -0.017. MS agar medium was prepared by adding 1.8 % agar to MSM broth. All media were sterilized at 121^o C for 15min before use.

Screening of Dye Degrading Bacteria

All soil samples were pretreated in such a way agitated in 100ml saline (0.9 %) at 140 rpm for 1hr. Then the soil was allowed to settle down for 30 min. For initial screening 20 ml of supernatant was used for the isolation of dye degrading bacteria was carried out by adding 20 ml of textile industries effluent / sewage or 20 ml supernatant of soil sample (diluted) to 100 ml of mineral salts medium (MSM) containing 50mg /l of Reactive Red -120 as sole source of carbon. All flasks were incubated at 35^{oC} for 30 days under static conditions. The incubated flasks were checked for the change in initial color intensity and turbidity periodically. The decrease in the color intensity and increase in turbidity of the MSM broth culture flasks were selected for initial screening of a dye decolorizing bacteria. Then 20ml of culture broth from the decolorized cultured flask was transferred to 100ml of fresh MSM broth containing each of dye and incubated for one week under static conditions. From the decolorized culture 0.5ml was taken out and inoculated onto the agar plates of MSM containing each of dye (50mg/l) and incubated at 35oC until prominent dye degrading bacterial colonies appeared. The obtained colonies formed were collected and further were checked for purity by streaking twice on MSM agar medium. After the second transfer of these colonies on to respective dye containing fresh MSM broth containing 0.05% to 0.1% yeast extract as co substrate. In 0.1% yeast extract showed maximum Decolorization so it had maintained the same throughout the experiment. Finally purified cultures were individually tested for their dye degrading capabilities in MSM broth under static condition several bacterial isolates capable of degrading the dye the taking less time were considered as potential strains and such strains were preserved at 20oC in 15 % glycerol (w/v) and used for further investigation.

Isolation and Characterization of Dye Decolorizing Bacteria

A total 10 morphologically different bacterial isolates were individually tested for their ability to decolorize dye in MSM broth. The dye decolorization experiments were performed in 250 ml Erlenmeyer flasks containing 100ml of sterilized MSM broth supplemented with yeast extract (0.1% w/v) and individual dyes under static conditions. 5ml sample was taken out aseptically and centrifuged at 10,000 rpm for 15 min. The cell free supernatant was used to check the percentage of decolorization of added dye using UV – Visible spectroscopy (syntonic Av -2700). The bacterial isolates showing maximum decolorization of dye within less time considered as most efficient and were used for further studies.

Preparation of Pre-Inoculum

Colony grown on mineral salt agar plate was added in the 100ml of nutrient broth solution incubated for overnight. From that 10 ml of culture was added to MSM containing Reactive Red -120 50(mg/l) dye supplemented with 0.1 % of yeast extract and incubated under static conditions checked for complete decolorization of dye. This decolorized culture was as pre - inoculum and used for the decolorization study.

Decolorization assay

For investigating the ability of the isolate for decolorization of Reactive Red -120, 10 % of bacterial inoculum was added to the 100 ml of MSM broth with 250 mg /l of Reactive Red -120 dye

(decolorization medium).The decolorization medium without culture served as control. The flasks were incubated at 350C. Every 4 hour 3 ml of the sample was drawn from each flask and analyzed for the dye content by determining the optical density at 520 nm in a uv - visible spectrophotometer for measuring the optical density the cells and other debris were removed by centrifugation (10,000 rpm for 10 min). A decrease in the optical density with time period is taken as an indicator of decolorization. To confirm the decolorization is due to degradation of dye not due to change in the pH of the medium and adsorption or absorption by change in the pH of the culture filtrate with HCl or NaOH. (Moosvi *et al*., 2007; Khalid *et al*., 2008)

Dye decolorization in MSM broth supplemented with yeast extract (0.1 % w/v) and Reactive Red 120 (250 mg/l). Complete decolorization of dye occurred within 24 hours of duration. Dye degradation was confirmed by the checking optical density at 520 nm for different intervals of time. The percentage of decolorization was calculated by following equation it is calculated at regular intervals (0, 2, 4, 8, 12, 16, 20 and 24 hrs) of incubation.

Calculation of % of Decolorization:

Percentage of degradation =

$$\frac{\text{Initial O.D} - \text{Final O.D} \times 100}{\text{Initial O.D}}$$

Results and Discussion

Characterization and identification of bacterial isolate

The result of cultural, morphological and biochemical characteristics are given in table 1.The colonies of isolated on nutrient agar medium observed to be round small size with regular margin, smooth and opaque in appearance. The Grams staining reveals that isolate is round shaped arranged in tetrads. Results of biochemical tests are listed in the table .based on all these tests, the isolate was tentatively identified as *Micrococcus Sps.* As per (Holt *et al., 1994).*

Table 1 Characterization and identification of bacterial isolate

Tests	Observation
A. Colony character	
Size	Small
Shape	Round with entire margin
Color	Yellowish
Texture	Smooth, opaque
B. Morphological characteristics	
Grams staining	Gram positive cocci
Motility	Non- Motile
Cell shape and arrangement	Round, arranged in tetrads
C. IMViC	
Indole	Negative
Methyl red	Negative
Voges proskaur	Negative
Citrate	Negative
TSI	Positive
D. Urease production	Positive

Optimization Of Biotic And Abiotic Factors:

The various biotic and abiotic factors were optimized for the maximum decolorisation by isolate.

Effect of Aeration

The effect of aeration or oxygen is one of the critical considered in decolorization of Reactive Red -120. The influence of the static and shaking condition on the decolorization performance of Reactive Red- 120 by isolate showed under static condition the decolorization of 82.85% was noticed while in shaking condition the decolorization only 54.34% was shown. Thus static condition was adopted for all further decolorization experiment.

Optimization of pH

The effect of pH on decolorization of reactive red 120 by our isolate was determined over a wide range of pH (5-12) and the maximum decolorization was at pH-9. At this optimum pH the strain showed 86.35% 0f decolorization of reactive red 120. In accordance with the earlier result on degradation of Reactive Red 195 by *Georgenia sp*s. CC NMPT-T3 showed constant decolorization from pH 5-8, maximum being at pH-7 (Madhuri *et al*., 2012). And another report which is showed on decolorization of azo dye reactive red BL by *Alcaligenes sps*. AA09 exhibited maximum decolorization rate at pH value near 7 (Pandey, *et al*., 2012). (Jain *et al*., 2012) reported that mixed locally isolated different *Bacillus sps* called bacterial mixed culture SB4 decolorize more than 85% in a wide range of pH 5-8.5 maximum at pH 7 and decolorization was decreased drastically when pH was increased to 10. (Mohan, *et al*., 2013) observed that the decolorization percentage was highest at alkaline pH for *Bacillus sps*. at pH 9 but in *Planococcus sps* decolorization percentage was found to be high in acidic pH(6). In the work of (Maulin P. Shah *et al*., 2013) maximum decolorization was observed in pH 8 but wide range of activity was seen between pH 6-10.

Optimization of Temperature

The rate of chemical reaction is the direct function of temperature. Bacteria require optimum temperature for growth. Since dye decolorization is metabolic process hence shift in temperature from optimum results into decrease in dye decolorization. The isolate showed maximum decolorization. The isolate showed maximum decolorization (85.62%) within 24hrs at 35^0C. Various microorganisms showed their survival at various temperature ranging from 25-50℃ (Madhuri Sahasrabudhe *et al*., 2012). The isolate showed decolorization of the dye within the temperature range of 28-50℃. Maximum decolorization was observed at 35℃. Similar reports have been reported by Joe *et al*., 2008 were *Clostridium bifermentous* SL-186 showed decolorization maximum at 35℃ and another report by S. Kannan *et al*., 2013 were the best decolorization was achieved at temperature 35℃ and 40℃ respectively. Bhatt Nikhil *et al*., 2012 reported that 37℃ temperature gave maximum decolorization by bacterial consortiumbut the isolate showed 80% of decolorization at 45℃. The activity decreased at 50℃ and 20℃. Sahasrabudhe Madhuri *et al*., 2014 reported that *Micrococcus glutamicus* NCCM 2168 can decolorize reactive red 195 at 37℃. *P. putida* decolorized methyl red in the temperature range of 34-37℃ (Mathur N. *et al*., 2013). Shah M.P. *et al*., also reported maximum decolorization of Remazol Black B by Bacillus ETL 2012 was observed at 37℃.

Effect of RR-120 concentrations on decolorising efficiency

In order to check the decolorization efficiency of our isolate, we exposed it for various initial concentrations of RR-120 (100-1000mg/liter). It was observed degradation efficiency of isolate was increased up to 200mg/L thereafter reduced gradually with increase of dye concentration and maximum decolorization of 85.54% was seen at 200mg/L. A survey of the literature suggests that increasing the dye concentration gradually decreases the decolorization rate, probably due to the toxic effect of dyes on the bacteria. It is also due to in adequate biomass concentration and blockage of active sites of azo reductase by dye molecules with different structure (S.R.Dave, *et al*., 2015). The different dye concentration exhibited varied range of effect on dye decolorization. There percentage ofdecolorization of Reactive Red 120 by isolated organism was carried out at different initial dye concentration (100mg-1000mg) it was observed that decrease in percentage of decolorization of Reactive Red with increase in concentration. Maximum decolorization was shown at 200mg dye concentration and gradually reduces the decolorization up to 60% at 1000mg dye concentration (Anjaneya, *et al*., 2011) reported 100%decolorization at 200mg/l and 62% for 1000mg/l dye concentration.

Effect of Salt Concentration

To check the effect of salt concentration on the decolorization of RR-120 by increasing 1% NaCl concentration at a time in the decolorization medium up to 5% was done .The decolorization was maximum at 2% thereafter it decreased gradually. Waste water from dyestuff manufacturing and textile processing industries shows presence of various acids, alkalis, metal ions and salt as impurities. Up to 15-20% salt concentration has been reported in this industrial waste water, because high salt needed for the dyeing process (Hazrat, 2010). So high salt concentration can inhibit bacterial growth. Generally a sodium concentration above 3% causes moderate inhibition of most bacterial activities. Thus, azo dye removal efficiency is inversely proportional to the salt concentration. In the present study the effect of NaCl concentration on the decolorization of reactive red-120 was examined the

isolate exhibits 80% of decolorization at 1% salt concentration & 65% of decolorization at 5% salt concentration which shows the maximum decolorization is at 1% salt concentration. Our results are similar with (Otukar et al.,2011) in which maximum decolorization of reactive red 120 by *Bacillus leutus* B1377 at 1% of salt concentration.

Optimization of size of inoculums

The effect of inoculums size (1-20%) on RR-120 decolorization by increasing percent of inoculums in the flask containing decolorizing medium. The maximum decolorization of 86.71% was shown in 24 hours with the optimum inoculums size of 10% was fixed for further experiment. Inoculums concentration depends upon the bacterial strain, In the degradation of red 3BN Bacillus cereus shown inoculums size 8% .(Praveen Kumar and sumangala.2012).(Rajee, *et al*., 2011) used 5% inoculum of *Micrococcus* sps to decolorize azo dye Orange MR.

Effect of additional carbon and nitrogen source and optimizing yeast extract

The effect of carbon sources like glucose, sucrose, maltose, lactose, starch on the decolorization of RR-120 by our isolates. It is revealed that all sugars are not influenced on decolorization of RR-120, however the maximum of 79.10% was of maltose and with 1g/L of different nitrogen sources like yeast extract, sodium nitrate, ammonium nitrate was added to the decolorizing medium with all optimized conditions to know the effect on the RR-120 decolorization efficiency of isolate. In yeast extract maximum decolorization of 85% was shown and finally yeast extract was efficient in enhancing the decolorization of RR-120. The decolorization efficiency was increased with increase in yeast extract concentration from 0.5-2gm/L. In 1g/L of yeast extract concentration showed maximum decolorization of 85.86%. Therefore it is used as a optimal additional nitrogen source. Carbon sources provide energy for the growth and survival of the bacteria and also transfer reducing equivalent to the dyes for azo bond cleavage (Soils, *et al*.,2012). The effect of different carbon and nitrogen sources exhibited varied range on dye decolorization. Among the carbon source our isolate showed highest degrading capability in maltose and among the nitrogen sources our isolate exhibited maximum degrading capability in yeast extract many reports are suggested the addition of yeast extract as nitrogen source increases the decolorization process using bacteria (Harshad Lade *et al.*, 2015) reported that by addition of glucose as carbon source reveled complete decolorization of Congo Red dye and RTE was achieved within 11 to 19 hours. He also reported that when glucose was added in combination with yeast extract and peptone the decolorization increased marginally compared to individual carbon or nitrogen source. Dr. P. Arulazhagan 2016 reported that cellulose and glucose were best suitable carbon source for the growth of B.subtilis. NH4OH and Na2NO3 were suitable as nitrogen source. Bacteria utilization of azo dyes as a source of carbon energy and nitrogen source (Mukund Thakur *et al*., 2012).

Conclusion

As we seen in the result the isolated strain based on their morphological , biochemical characteristics is tentatively identified as *Micrococcus species* .It has the ability to decolorizethe Reactive Red-120dye, has been studied by performing various parameters like static conditions shows 82% of decolorization,optimum pH was 9 and temperature was 35°C,maximum decolorization was shown at 200mg/L dye concentration and up to 2%ofsaltconcentration tolerance and the inoculum size is 10ml,additional nutritional sources were selected in carbon source maltose shown maximum decolorization and in nitrogen yeast extract show maximum decolorization.. Hence,overall result suggest the isolate can be used for the biological treatment of industrial effluent wastes containing dyes.

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Quick Response Code	Subject: Biotechnology
DOI:10.22192/ijarbs.2019.06.05.014	

How to cite this article:
Madhuri R Basutkar , Aditi, Nimrah Fatima, Prashantkumar S Chakra, Ruhee Mehtab, Shweta Swami, Shuwana Shermeen, Vishwaraj, and C.T. Shivannavar. (2019). Decolorisation of Reactive Red-120 Azo Dye by *Micrococcus* Sps. Int. J. Adv. Res. Biol. Sci. 6(5): 118-125.
DOI: http://dx.doi.org/10.22192/ijarbs.2019.06.05.014