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Molecular and Biochemical Identification of Reactive Red-11 Decolorising *Klebsiella oxytoca* CMGS-3 Bacterial Strain and Study of Toxic Effect on Plants

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A B S T R A C T

The tentatively identified bacterial strain was showed decolorisation of 82.3% in mineral salt of medium with 100mg/L Reactive red-11 without any external nutritional source within 72 hours. Finally optimization done at pH-8, temperature -35ºC and with 0.1 % (1gm/L) yeast extract and dye concentration was 200mg/l and decolorisation confirmed around of 94% within 16 hours of incubation. Further work carried on The identification of bacterial isolate and identified as *Klebsiella oxytoca* CMGS-3 by16s rRNA technology (Gene bank accession num- KT602858), and various biochemical and sugar utilization tests were done, finally complete decolorisation confirmed by the FT-IR analysis, and green house studies were done to check the toxic effect of dye; here the decolorized and untreated dye used to study toxic effect on crops which were widely grown agriculture crops and CMGS-3 Strain showed better results in germination as well as plant growth. Through these results, it concludes isolate CMGS-3 was the better candidate for the decolorisation of wide range of reactive dyes and it is preserved in IMTECH, (Chandigarh) with the MTCC num-12532 for the usage of waste water treatment.

Introduction

Colors are the part of life, natural and synthetic man made chemical colors is the types of it. Human creates obstructions in nature in the form of pollution, industrial pollution playing major part of it. Pollutions are various kinds and out of synthetic textile dye pollution taken a leading role. Textile Dyes industries produce huge amount of synthetic dyes wastes. In Asia, India may become second major contributor of textile waste water discharging (Verma *et al.*, 2012). Worldwide synthetic dye production is around $1x10^{-6}$ tones (Pandey *et al.*, 2007). More than 10000 different kinds of dyes are available in world (Kadam *et al.*, 2011). These dyes are carcinogenic, toxic, and mutagenic in nature (Shah *et al.*, 2013). Among all textile dye, azo dye constitute 70% worldwide (Govindwar *et al.*, 2011). In textiles dyes production 60-70% azo dye manufacturing takes place. Structurally these contains N=N bonds, attached to a benzene ring, various other

compounds like SO_3H, SO_2NH_2, attached to a aromatic nucleus which makes into recalcitrant (Jain *et al.,* 2012). Dyes when come across with the water, its quality and parameters changes occurs, increases BOD, COD, TDS, DO, PH of water and dyes contain complex hazardous chemicals, they are chlorine groups, acidic group, heavy metals and other harmful chemicals (Saraswathy and Balakrishanan, 2009: Abraham and Muraleedhara, 2014).

There should be strict legislation to control of discharging of dyes. Various physicochemical treatments are available like flocculation, adsorption, oxidation, but failed to convert dyes to non toxic as end product (Metwally and Elshora, 2008). In India textile industries considered as huge amount generators of liquid effluents, which are contaminated with poisonous dyes, it is estimated from other industries textiles production 14%., and 27% its export earnings (Saratale *et al.,* 2011). Recently microbial degradation taken a leading role because of its cheapest, effective, less time consuming and eco friendly in nature (Kalyanee *et al.,* 2007). Many microbial species like fungi, actinomycetes, algae, bacteria are reported in the effective degradation of synthetic dyes (Asad, *et al.,* 2008). The fungal decolorisation is slow and time consuming (Tapia and Tusell, 2011).

In contrast to other microorganisms bacterial degradation is faster (kaushik *et al.,* 2011). Bacterial decolorisation process is faster, due to its replication cycle and gene arrangements (Vigneswaram *et al.,* 2010). Bacteria perform degradation in single or mixed cultures (cherriaa *et al.,* 2012). In this study we isolated a efficient dye degrading strain, which was used in the degradation of reactive red-11, and degraded metabolite of effluents tested on agriculturally used crops *Tricicum aeticum* and *Vigna Radiata* for the duration of 15 days, checking of effect on the dyes, seeds and plants shown better results in the germination as well as propagation of plants (Swapnil *et al.,* 2011).

Materials and Methods

Dyes and chemicals

A total of six Reactive Azo dyes were procured from Sigma Aldrich (U.S.A), Heena and Colorise textile industries (Gujarat, India). They are Reactive Blue-4, Reactive Yellow-86, Reactive Navy Blue-59, Reactive Orange-16, Reactive Violet-1, and Reactive Red-11. All these dye were industrial grade and widely used in the textile industries. Out of these Reactive Red-11 (RR-11) widely used azo dye and selected for the dye decolorisation studies. All required chemicals, solvents and reagents used in the study were of analytical grade and were procured from, reputed firms (Hi-Media Laboratories Pvt. Ltd., Mumbai; S.D. Fine Chemical Ltd., Mumbai). For the biochemical profiling Hi-Carbo kit used which was procured from Hi-Media and TLC plates from Merck India (Merck Specialties Pvt. Ltd., Mumbai).

Collection of Samples

The soil and effluent samples were collected from the textile industrial area and textile effluent treatment unit, MIDC, Solapur, Maharashtra. Samples including untreated textile mill effluent, sludge soil, dye contaminated soil, greasy soil, colored charcoals from the textile industry, untreated synthetic dye contaminated water. Further some samples were collected from in and around of Kalaburagi, like dye contaminated soil from the M.S.K. Mill area, sewage samples, garbage soil, garden soil. Soil samples from Sharanabasaveshwara lake, Ganesh statue making place (colored soil), market area, dairy unit were also collected. All the samples were collected in a clean

sterile bottle and other containers and brought to the laboratory. All the collected samples were processed immediately in the labs if delayed stored in the refrigerator (4^0 C).

Preparation of Media and Reagents

The mineral salt medium (MSM) was prepared as per (Brilon *et al.,* 1981) with some modification. 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. Further, MSM was blended with different concentrations of RR-11or RO-16 separately and used as decolorizing medium (DM) throughout the study and un-inoculated flasks were also incubated as control. The MS with 1.8% agar was used for isolation and maintenance of pure culture. The media were sterilized at 121^0 C for 20 min before use.

Isolation and screening for dye decolorizing bacterial isolates

For the isolation process the protocol referred which is prescribed in Anjaneya *et al.,* (2011). 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 RR-11 decolorizing microorganisms. Twenty ml of supernatant was inoculated to 100 mL Mineral Salt Medium (MSM) containing 50 mg/L RR-11 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 RR-11 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 RR-11 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 RR-11 slants and after growth two slants were stored at 4^0 C after adding 25% of sterile glycerol on the culture surface and one slant used for the further study.

Screening for dye decolorizing potential bacterial isolates

Among the bacterial strains isolated from the decolorized flask a total of 30 morphologically different bacterial strains were individually tested their ability of RR-11 decolorisation. The mineral salt medium was

blended with RR-11 (50 mg/L) and sterilized at 121^0C for 20 min. The pure bacterial isolates were grown in nutrient broth for 18 hrs, than 10 mL of culture was inoculated into 100 mL of DM and were incubated at 35^0 C without shaking. The decolorization in each flask was routinely checked by measuring the dye concentration in the supernatant of culture at 540 nm for RR-11.

The flasks showing maximum decolorization of RR-11 within less incubation time were selected for the further study.

Characterization and identification of dye decolorizing bacterial isolates

The isolated bacterial strains were identified up to the genus level by comparing their cultural, morphological, staining, biochemical and physiological properties with characters mentioned in the Bergey's manual of systematic bacteriology (Holt *et al.*, 1994).

Confirmation of bacterial isolates by 16s rRNA sequencing and phylogenic analysis

The tentatively identified three potential RR-11 decolorizing bacterial strains were identified and confirmed up to species level by 16s rRNA sequencing method. Pure culture from a single colony was selected and sent to Royal Life Sciences Pvt. Ltd., Hyderabad, India for 16s rRNA sequencing and identification. Using 16s rRNA sequencing data evolutionary analyses were conducted in MEGA-6 software. The sequences of all three isolates were deposited in NCBI with their accession numbers and cultures were deposited in IMTECH, Chandigarh with (MTCC) number (Fig. 1).

Klebsiella sp. CMGS-3- identified as *Klebsiella oxytoca* CMGS-3 (bp-1589) NCBI accession number-KT602858 and MTCC-12532.

Analysis of reactive red-11 degraded products at different intervals of incubation period.

UV-Visible Spectroscopy (UV-vis), Fourier transform infrared spectroscopy (FT-IR) were used to understand the biodegradation pattern of RR-11 by the isolate CMGS-3 with optimized parameters under static condition.

UV-Vis spectroscopy

The supernatants of different intervals of incubated DM with RR-11 and CMGS-2 were subjected to scan between 190 to 900 nm UV-Vis spectrophotometer results are shown in Figure 2.

A single peak at 540 nm corresponding to λ_{max} of the dye and two intense peaks at 250 nm and 325 nm corresponding to phenyl and naphthol rings of RR-11 respectively.

With increase in incubation time the peak height at 540 nm goes on decreased and disappeared after 16 hrs of incubation indicates complete decolorisation of added RR-11 (Fig. 3).

Fourier transform infrared spectroscopy (FT-IR) analysis.

To analyze degradation capacity of isolates extracted metabolites from complete decolorization of RR-11 and RO-16 were sent to the Sipra Pvt. Labs, Hyderabad. The FT-IR analysis was done by using Fischer Scientific (Nicolet, iH5) Spectrophotometer with 32 scan speeds the samples were mixed with spectrophotometrically pure KBr in ratio (5:95).

The pellets were fixed in sample holder and analyzed. The spectra in the IR region between 400–4000 cm^{-1} were used to see types of metabolites present.

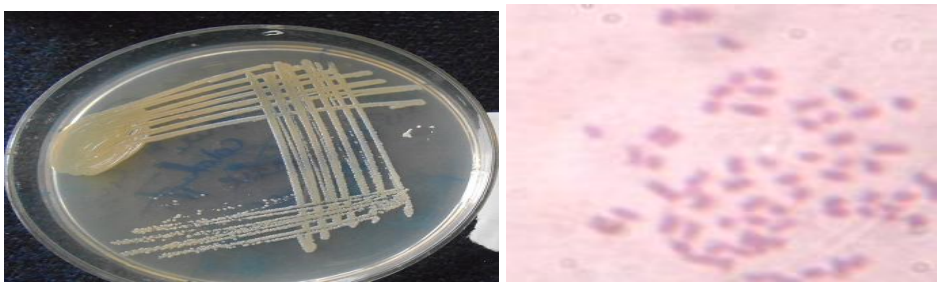
Table.1 Cultural, morphological and biochemical characteristics of bacterial isolate strain CMGS-3

Tests	Observation
A. Colony characteristics on nutrient agar	
Size	Medium, Mucoid.
Shape	Entire
Color	Pink
B. Morphological characteristics	
Grams staining	Gram negative bacilli
Cell shape and arrangement	Thick and short rods arranged singly
Motility	Non motile
Spore staining	No spores
C. Carbohydrate utilization	
Glucose	Acid
Sucrose	Acid
Lactose	Acid
Mannitol fermentation	No
D. IMViC tests	
Indole	- ve
Methyl Red	+ ve
Voges Proskaur	+ ve
Citrate	+ ve
E. Urease production	-ve
F. Catalase test	-ve
G. Gelatin hydrolysis test	- ve
H. Nitrate reduction test	+ve
I. Starch hydrolysis test	+ve

Biochemical profile tests using (Hi-Carbo™ kit) for bacterial isolate CMGS-3

Tests	Observation
Lactose	+ve
Xylose	+ve
Maltose	+ve
Fructose	+ve
Dextrose	+ve
Galactose	+ve
Raffinose	+ve
Trehalose	+ve
Melibiose	+ve
L-Arabionose	+ve
Mannose	+ve
Inulin	+ve
Sodium gluconate	-ve
Glycerol	+ve
Salicin	+ve
Dulcitol	+ve
Inositol	+ve
Sorbitol	+ve
Mannitol	+ve
Adonitol	+ve
Arabitol	+ve
Erythritol	+ve
Alpha-methyl-D-glucoside	+ve
Rhamnose	+ve
Cellobiose	+ve
Melezitose	+ve
Alpha –methyl-D-mannoside	-ve
Xylitol	-ve
ONPG	+ve
Esculin hydrolysis	+ve
D-arabinose	+ve
Citrate utilization	+ve
Malonate utilization	+ve
Sorbose	+ve

Figure.A) Growth of CMGS-3 on MSA containing RR -11.
B) Grams staining image of CMGS-3



Klebsiella sp. CMGS-3 (1589 bp)

CTATCAGTACGTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTCGAGATTGAACGCTGGCGGCAGGCC
TAACACATGCAAGTCGAACGGTAGCACAGAGAGCTTACGACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGACGCGGTGGAGCATGTGGT
TTAATTCGATGCAACGCGAAGAACCGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGG
ACACGCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGCGCTTCGGGCCTCTTGCCATCGGATG
TGCCCAGATGGGATTAGCTTGTAGGTGAGGTAACGGACGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAACGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAACGGTCCCGCA
ACGAGCGCAACCCTTATCCTTTGTTGCCAGCCGGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
GCGCAAGCACGGATTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGCGCTGATGCAGCCATGCCGCGTGT
ATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGACGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTA
CACACGTGCTACAACGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACACGTGGCATA
TACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGCGCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGG
GTGCAAGCGTTAATCGGAATTACGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGCGACTGGGCGTAAAGCGCAC
GCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAAACGAAGTCGGAATCGCTAGTAATCGTGGATCAGACGCCTGGGA
ACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGACGATGCCACGGTGAATACGTTCCCGGGCCTT
GTACCGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGACACCGCCCGTCACA
CCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTACGACAAAGACTGACGCTCAGGTGCAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAACGACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGAAGTCGCGGTCCACGCTGTAAACG
ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCACGTAACACGTAACGCGTAA

Figure.1 16s rRNA sequences of CMGS-3 isolate and its Phylogenetic position

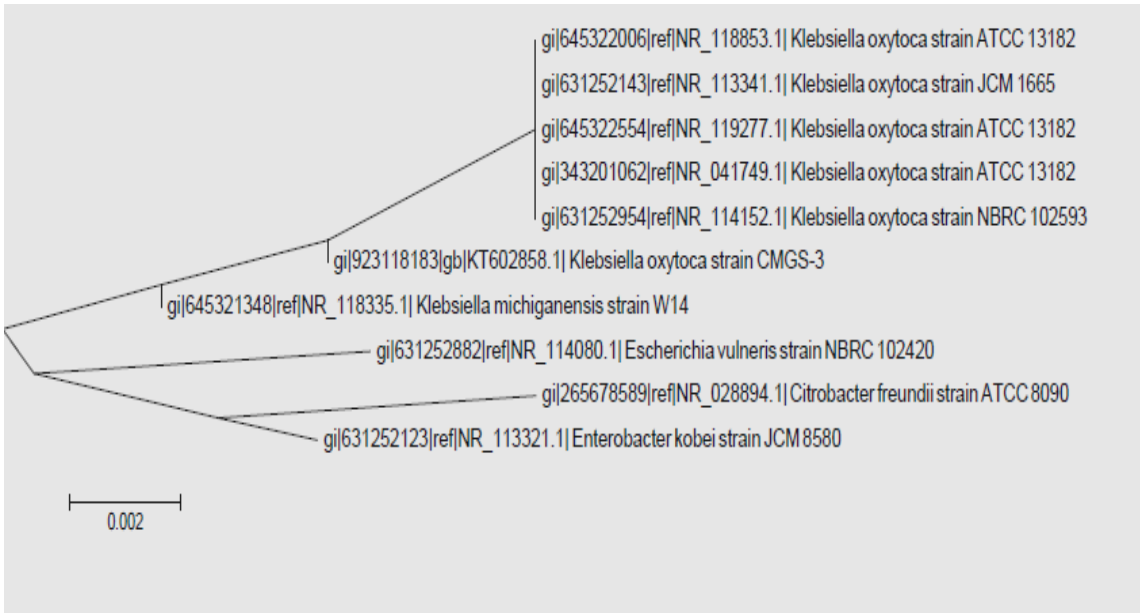


Figure.2 Control RR-11

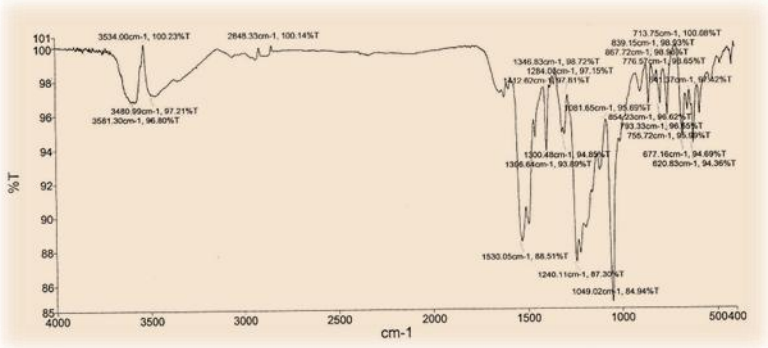
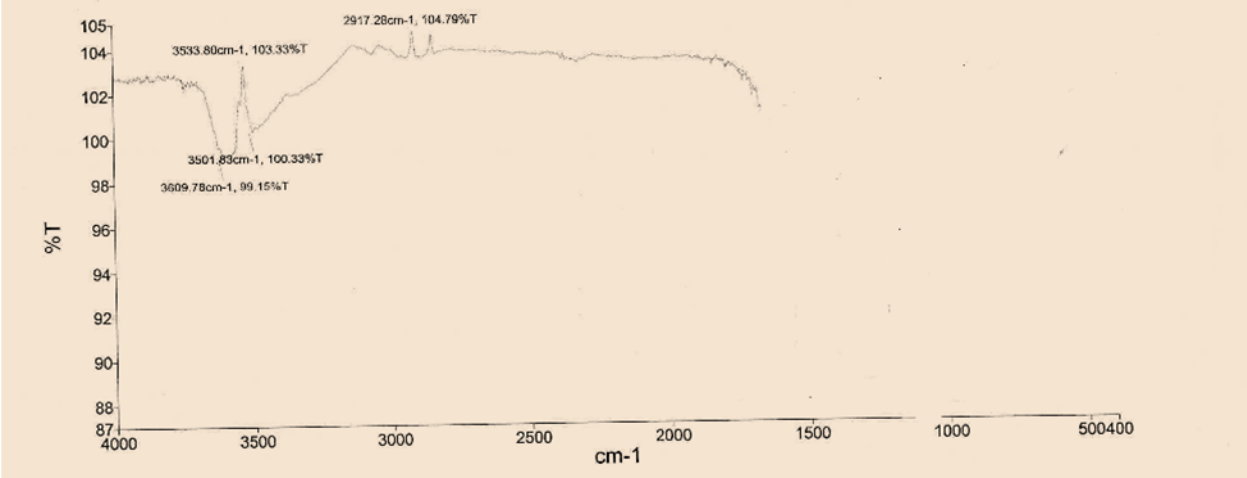


Figure.3 16 Hours decolorized metabolite



Study of toxic effect of treated and untreated effluent on seed germination and growth of plants

Tests were carried out on two kinds of seeds commonly cultivated in India. Two widely cultivable plant species are (*Triticum aestivum*) Wheat, (*Vigna radiata*) Mung bean, were selected for the toxicity study and checking the effect on the seeds germination and plant growth up to15 days. Ten seeds of each plant were taken in six sterile petri plates containing sterile cotton first one is wetted with tap water, the second with treated effluent and third with untreated effluent for Wheat, similarly petri plates used for the Mung grains and were incubated in dark at 30-35°C for 48 hrs and then recorded the number of seeds germinated in each plate and calculated percentage of germination in each plate in treated and untreated seeds of both kinds of grains. Further, seeds were sowed in soil and toxicity was measured by pouring 10 mL of sample (tap water as control, untreated and treated reactive red-11) daily. Calculated growth of plants in terms of lengths of shoot and root in centimeters (cm) after 15 days and compared with that of control to evaluate the effect of seed germination was calculated following formula (Chetana *et al.*, 2016; Anjaneya *et al.*, 2011).

Germination (%) = No. of seeds germinated × 100 / No. of seeds sowed.

Biodegradation of reactive red-11

Structure and properties of Reactive Red-11 (RR-11)

Reactive Red-11 is a synthetic dye, mainly used in the dying of cotton, viscose fabric, silk and in printing. Its properties are sparingly soluble in water and methanol. Structurally made up of single azo (N=N) bonded chromopheric group. This having Chlorine, SO_3, NH, NaOOC, as reactive groups attached with azine, naphthalene and benzene rings to form complex structure so, it is called as polycyclic aromatic azo dye and is not easily degraded in the environment.

Chemical Assessing System Registry Number (CASR): 12226083.

Manufacturing method - 4-Aminobenzoicacid diazo are maintained in alkaline conditions and 4-amino-5-hydroxynaphthalene 2,7-disulfonic acid coupling, with 2,4,6 Trichloro 1,3,5-triazine condensation. The molecular formula is $C_{20}H_9Cl_2N_6Na_3O_9S_2$ with molecular weight of 681.33 Daltons and λ_{max} of 540 nm.

Biodegradation studies by FT-IR

Control reactive red-11, 23 different peaks were observed, spectroscopy run from 400 to 4000cm^{-1} wavelength. The control spectrum of reactive red -11 was found 3581.30cm^{-1},3535.00 cm^{-1},3480.99 cm^{-1}showing the intermolecular hydrogen bonding, and –OH Aromatic, O-H Streching.2848.33cm^{-1}peak for N-H stretching amines. 1530.05cm^{-1} for C=N Stretching Azo Group, 1081.65 1049.02 for S=O stretching group, 620.83.,677.16,839.15, 867.72, 776.57, 793.33,854.23, 755.72 these peak shows dye is in aromatic in nature and C-CL Stretching respectively. and 16hours dye degraded sample three peaks observed peaks they were 988.02.1409.56.3433.91 there was no aromatic compound peak, N=N regions peaks also not seen, these study shown complete decolorisation of RR-11(R.silverstein, *et al.*, 1998: Wojnárovits *et al.*, 2005; Yingling, *et al.*, 2008 ; Rajeshwari *et al.*, 2011).

Study of dye toxicity effect on seed germination and plant growth

Effect of treated and untreated reactive red-11 on - *Tricicum aestivum* and *Vigna radiata.*

Decolorized dye and untreated reactive red-11, were selected for the study of dye toxicity on crops, two widely agricultural crops *Tricicum aestivum* and *Vigna radiata* were selected. Results compared with water as control. *T. aestivum* grown in the normal water showed 100% of seed germination and with shoot length of 15 cm and root length of 2.3 cm. Results of untreated effluent showed 60% of germination, shoot length 8.1 cm and root length 0.6 cm. Further, *T. aestivum* grown in the treated effluent showed 90% of germination, shoot length of 14.1 cm and root length 2.0 cm Similarly results of effect of treated and untreated dye sample effluent on *Vigna radiata* grown in the normal water showed 100% of seed germination with shoot length 11 cm and root length 2.0 cm and grown in the untreated effluent showed 55% of seed germination, shoot length 8.1 cm and root length 0.4 cm. However, with treated sample showed 90% of germination, shoot length 12.1 cm and root length 1.8 cm.

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