

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.511.067>

**Biodegradation of Reactive Red-11 by the Isolate
Enterococcus casseliflavus CMGS-1 Strain**

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A B S T R A C T

Keywords

Potential,
*Enterococcus
casseliflavus*,
FT-IR spectrum,
MTCC, IMTECH.

Article Info

Accepted:
24 October 2016
Available Online:
10 November 2016

The study was designed to isolate a potential dye degrading novel isolate, which is capable degrading wide range of textile dyes. In this direction a isolate identified as *Enterococcus casseliflavus* strain isolated from textile treatment unit, showed decolorisation of 98.91% in mineral salt medium blended with 200mg/L reactive red-11 as sole source carbon within 72 hours of duration, and to optimized the decolorisation capacity of isolate, various abiotic and biotic parameters were framed, finally organism showed 96.56 % decolorisation of 400mg/L reactive red-11, at pH-9, temperature 40°C, inoculum concentration 5ml, and yeast extract 1gm/L at 16 hours of duration. The complete degradation was confirmed by FT-IR spectrum analysis and strain was preserved in MTCC (IMTECH) with MTCC no:- 12538.

Introduction

Pollutions are the insertion of contaminations, in the natural environments leads to various obstructions, in water pollutants synthetic dye pollution emerging rapidly. In Asia, India may become second major contributor of textile waste water discharging (Verma *et al.*, 2012). The basic structure constituent of dyes are contains n=n bonds, attached to a benzene ring, and various other compounds like SO₃H, SO₂NH₂, NO₂ Attached to a aromatic nucleus (Jain *et al.*, 2012). Various physical, chemical methods like reverse osmosis, Fenton's reagent, chemical flocculation, ion exchange, coagulation, are available but these methods are expensive

leads secondary sludge generation and complete degradation cannot be achieved (Jadhav *et al.*, 2012). 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). The ultimate solution we will get from microorganisms, these are the natural cleaners of the nature. In these direction we selected the potential strain which having the capacity to degrade reactive red-11, confirmed by the FT-IR analysis.

Materials and Methods

Sources of sample: Soil and effluent samples were collected from the textile

industrial areas and textile dye treatment unit (MRDC) of Sholapur Maharashtra.

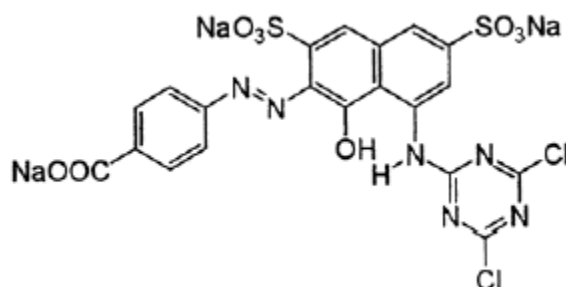
Dye:- Reactive red-11 procured from the Colorise, Heena textile industries. Gujarat (India).

Information and molecular Structure of Reactive red-11

Presently work carried in the degradation of synthetic Reactive red -11 dye, used mainly in the dyeing of cotton, viscose fabric and silk, in printing. Its properties are- single azo

(n=n) bonded chromopheric group, having Chlorine, SO₃OH, NO₂, as reactive group attached with benzene rings. It is having molecular weight—681.33 with a λ_{max} 540nm, molecular formula: C₂₀H₉Cl₂N₆Na₃O₉S₂. CAS Registry Number: 12226083. Manufacturing Method is: - 4Aminobenzoicacid diazo, in alkaline conditions and 4Amino5hydroxynaphthalene2, 7disulfonicacid coupling, again with 2, 4, 6Trichloro1, 3,5triazine condensation.

Structure of reactive red-11



Chemicals

The chemicals and solvents used in this work were of analytical grade and procured from standard companies like HI-media Pvt.Ltd. Mumbai. Sugar utilization tests were performed by using Hi-Carbo kit.

Mineral Salt Medium (MSM)

The mineral salt medium (MSM) broth was prepared by adding Na₂HPO₄.2H₂O -12.00 g, KH₂PO₄ -2.00 g, NH₄NO₃ -0.50 g, MgCl₂. 6H₂O -0.10 g, Ca(NO₃)₂. 4H₂O - 50.00 mg, FeCl₂.4H₂O - 7.50 mg to 1000 ml of distilled water and to this 10 ml of trace elements solution was added before adjusted the pH 7.0. Trace elements solution was prepared by adding FeSO₄.7H₂O -0.10 g, ZnSO₄. 7H₂O- 0.10g, CuSO₄.5H₂O- 0.1g, CaCl₂.6H₂O - 0.1 g, MnSO₄.H₂O -0.17 g to

1000 ml of distilled water and 10 ml of trace element solution added MSM, finally media were sterilized at 121° C for 15min before use (Usha *et al.*, 2012).

Preparations of samples and isolation, screening of RR-11 decolorizing bacterial strain

10grams of soil sample weighed and water 10 ml of water sample poured into the 100ml of 0.8% saline solution and kept on the rotary shaker at 150 rpm for the duration of one hour. After one hour flasks were taken the rotary shaker, left for the soil settlement. And carefully 20ml of supernatant was added in the 50mg/L RR-11 +MS Broth ,kept for the observation in the static condition routinely decolorisation were checked at 540nm using UV-VIS Spectrophotometer, flasks showing more

than 50% of the decolorisation supernatant transferred to the fresh MS Broth+50mg/L RR-11 dye as the sole source of carbon. The flask showing fastest decolorisation were selected and loopful of culture were streaked on the MSM+50mg/L RR-11 dye as the sole source, the colonies were showing zones were selected for the screening,

One set of pure culture of dye decolorizing bacterial strains were selected and sub cultured on nutrient agar slants and stored in refrigerator with 25% sterile glycerol. Another set was used for the screening of the decolorisation pure culture was grown in the nutrient broth and 10ml of NB was added to the 100ml MS Broth+ 100mg/L RR-11 observation was done routinely.

Decolorisation assay:- It was studied in two different ways one with decolorisation in dye as the sole source of carbon and with 1gm/L yeast extract as the additional nutrient source. The bacterial isolates were showing the fastest rate of decolorisation within shorter duration of time was selected, in MSBroth+100mg/L RR-11, out of other isolates, a bacterial strain designated AS CMGS-1, was selected which was decolorizing the 200mg/L RR-11 within 72 hours, and in yeast extract was 400mg/L was the optimum, The flasks were incubated at 35⁰ C. To determine rate of decolorization every 4 hour 3 ml of the sample was drawn from each flask and centrifuged at 10,000 rpm for 10 min then supernatant used for taking optical density at 540 nm in a UV-Vis spectrophotometer. A decrease in the optical density compared to control was taken as an indication of decolorization The decolorization medium without culture served as control, in 16 hours of incubation isolate CMGS-1, showing 95.9% of decolorisation which is selected by final conformation using To know decolorization is due to change in the pH, the culture

filtrate was checked for alteration in the initial pH, if so; again check the color change in supernatant by adding HCl or NaOH. The decolorization is due to adsorption was tested by dissolving the culture pellet in the solvent. Similarly absorption was performed by analyzing the dye in the cell lysate.

Decolorization procedure: Dye decolorisation in MS broth supplemented with yeast extract (0.1% w/v) and reactive red-11 (400mg/L) complete decolorisation of dye occurred within 16 hours of duration. Dye decolorization was confirmed by the checking optical density at 540 nm for different intervals of time. The percentage of decolorisation was calculated by following equation.

Calculation of % decolorization

Percentage of decolorization = $\frac{\text{initial O.D} - \text{final-O.D}}{\text{initial O.D}} \times 100$

Optimization of various abiotic and biotic factors for maximum dye decolorisation by isolated bacterium

The abiotic factors including pH, temperature and aeration were optimized. Using wide range of pH from 4-12 with an increase of pH values 1 at a time and temperature in the range from 20-50⁰C with 5⁰C (interval). For the aeration flasks were incubated on incubator shaker with a speed of 120 rpm and for static condition flasks were incubated in normal incubator at 35⁰C. After knowing the optimum pH and temperature bacterial inoculums size was measured by adding 5 to 20% (5, 10, 15, 20) to 100 ml of DM.

The effect of additional nitrogen nutrients on the dye degrading efficiency of isolated bacterium with all optimized conditions was

performed by adding 1% of selected organic and inorganic nitrogen and different carbon sources to DM culture. The nutrient source showed maximum percent decolonization of RR-11 and was more than the control (culture without the additional nutrients) was selected and used for the determination of optimum concentration required for maximum decolorization by isolate. Determination of minimum yeast extract concentration required to optimize the decolorisation efficiency of an isolated bacterium was performed by adding 0.1 to 2% of yeast extract with an intervals of 0.5 %.

Morphological Identification

Loopful culture of isolate CMGS-1 streaked on nutrient agar plate, after 24 hours of incubation, colonies were selected for the morphological and colony characteristics studies they are size, shape, motility, gram staining, etc

Biochemical Identification

various biochemical tests were performed to characterize the isolate CMGS-1, tests were: carbohydrate utilization tests, IMViC tests, Urease production, catalase, gelatin hydrolysis, nitrate reduction, starch hydrolysis, tests as prescribed Experiments in Microbiology, Plant Pathology and Biotechnology, (K. R. Aneja New Age International Microbiology, 2003) and Bergey's Manual of Systematic Bacteriology, second edition.

Sugar Utilization Tests

various 32 Sugar (carbohydrate) utilization tests were performed by using HI-Carbo kit (Himedia). 18 hours grown culture of isolate CMGS-1 (20µl using sterile pipette) was added in the each vial

of sugar utilization kit, kept for the 24 hours of incubation, results were noted down.

16s rRNA Identification

Pure bacterial colonies were selected for the sequencing, sent for the identification, at Royal Life Sciences Pvt. Ltd. Hyderabad, India. The alignment of the sequences was done using CLUSTALW program V1.6 at European bioinformatics site (<http://www.ebi.ac.uk/Tools/msa/>). The sequence was refined manually after crosschecking with the raw data to remove ambiguities and submitted to GenBank. Evolutionary history checked by To see the Phylogenetic position of bacterial isolate using the neighbor-joining method (Saitou *et al.*, 1987). The optimal tree with the sum of branch length = 0.28801765 is shown. The Phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takazeki *et al.*, 1995). The clock calibration to convert distance to time was 0.02 (time/node height). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the Phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004). and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. Evolutionary analyses were conducted in MEGA6 software (Koichiro Tamura *et al.*, 2013).

FT-IR Analysis Procedure

To analyze degradation capacity of isolate CMGS-3, Reactive red-11,, and a 16 hour (400mg/L RR-11) degraded sample were sent to the Sipra Pvt Labs, Hyderabad and FT-IR analysis was done by using Fischer Scientific (Nicolet, iH5) Spectrophotometer

and IR region of 400–4000 cm^{-1} with 32 scan speed.

Preparation of sample: 16 hour degraded samples were centrifuged at 10000rpm for 10mins duration. Above supernatant was taken, and saturated with equal volume of ethyl acetate and separated the organic phase, kept at 45.^c in thermostat incubator and observed until complete evaporation of ethyl acetate extracts. Complete air dried metabolites dissolved in methanol. After evaporation of methanol in degraded metabolites weighed and sent to the FT-IR analysis, with reactive red-11 dye as control for comparison (Bheemaraddi *et al.*, 2014).

Study of phototoxic effect on the plants

Two widely grown agriculture plant species, *Tricicum aeticum*, *Vigna radiate*, were selected for the phototoxic study, conducted for the upto 15 days of duration and observed results of the germination and plants growth. Phototoxic study divided into two ways, water as the control, through these control water germination and plant growth compared with untreated and treated metabolites of waste water sample (centrifuged at 10000rpm). Results were noted down, treated metabolites. Shown excellent results in the germination and plants growth. By these it is concludes that isolate CMGS-1 able to degrade dye completely, can be used for in the treatment of synthetic waste water treatment.

Results and Discussion

Isolate CMGS-1 was tested due to the decolorisation ability, it was isolated by the textile dye treatment solapur Maharashtra, shown 98.91%, decolorisation in 200mg/L RR-11 within 72 hours. In yeast extract was 400mg/L was the optimum in 16 hours of the incubation.

Isolation and Characterization of Reactive Violet-1 decolorizing bacteria

The colony characteristics were studied on the nutrient agar medium, and gram positive bacteria confirmed by the gram staining process various biochemical, sugar utilization testes and 16s rRNA analysis done.

Optimization of abiotic and biotic parameters of *Enterococcus casseliflavus* strain CMGS-1

Decolorization assay for determination of dye decolorizing efficiency isolate *Enterococcus casseliflavus* strain CMGS-1

Utilization RR-11 by an isolate CMGS-1, as a sole carbon source in mineral salt medium and with yeast extract *Enterococcus casseliflavus* strain CMGS-1

It shown effective decolorisation in the RR-11(200mg/L) within 72hours of incubation and in the sam Concentration 0.1% yeast extract (1gm/L) was added the optimum Decolorisation was noticed around (400mg/L) 96.60% with 16 hours of incubation under static condition. According (Jain *et al.*, 2012; P. S. Patil, *et al.*, 2008) the decolorisation depends upon the structure of Dyes and metabolites produced during the decolorisation procedure. To enhance the decolorisation Capacity of isolate various abiotic and biotic parameters were programmed

Effect of static and shaking conditions:-

isolate CMGS-1 showed effective decolorisation in static condition. It static condition the % of decolorisation was 97.05%, and shaking it was 50.56% of decolorisation. For decolorisation isolate CMGS-1 requires static condition and it is kept constant for Upcoming parameters.

Optimization of pH concentration

pH is considered as the essential, isolate CMGS-1 shown decolorisation in all range of pH conditions, and effective decolorisation was observed at the pH-9:-96.96. alkaline pH is the better condition for the effective decolorisation. Similar with our results (Imran *et al.*, 2014) showed that *Shewanella* sps IFN4 decolorized mixed azo dyes in the pH range of 5-9. Decolorisation capacity depends upon organisms.

Optimization of temperatures

Isolate CMGS-1 is shown effective decolorisation in thermophilic range that is at 45°C:-97.2 % decolorisation. Similar with the results (Ali *et al.*, 2010) stated that the mesophilic range is 25⁰ – 45⁰C suits for waste water treatment plants including dye effluents treatment plants in tropical conditions.

Table.1 Morphological and biochemical characteristics of *Enterococcus casseliflavus* strain CMGS-1

Tests	Observation
A. Colony character	
Size	Small
Shape	Entire
Color	White
B. Morphological Characteristics	
Grams staining	Blue colour cocci (Positive)
Motility	Non motile
Cell shape and arrangement of spore	No spore former
C. Carbohydrate	
Glucose	Acid production
Sucrose	Acid production
Lactose	Acid production
Mannitol fermentation	+ve
D. IMViC	
Indole	- ve
Methyl Red	+ ve
Voges Proskaur	+ ve
Citrate	- ve
E. Urease production	+ve
F.catalase	+ve
G. Gelatin hydrolysis	- ve
H.Nitrate reduction	+ve
I Starch hydrolysis	-ve

Table.2 Sugar utilization tests

Tests	Observation
Xylose	+ve
Maltose	+ve
Fructose	+ve
Dextrose	+ve
Galactose	+ve
Raffinose	+ve
Trehalose	+ve
Melibiose	+ve
L-Arabinose	+ve
Mannose	+ve
Inulin	+ve
Sodium gluconate	+ve
Glycerol	+ve
Salicin	+ve
Dulcitol	-ve
Inositol	-ve
Sorbitol	-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
malonate utilization	-ve
Sorbose	-ve

Fig.1 Phylogenetic tree of *Enterococcus casseliflavus* CMGS-1

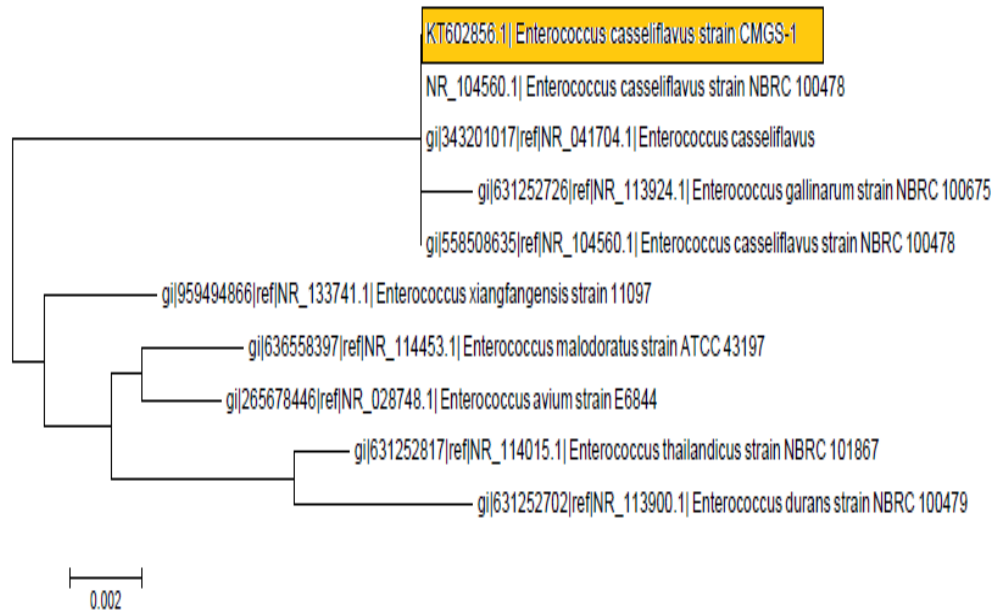


Fig.2 Colony characters on nutrient agar plate

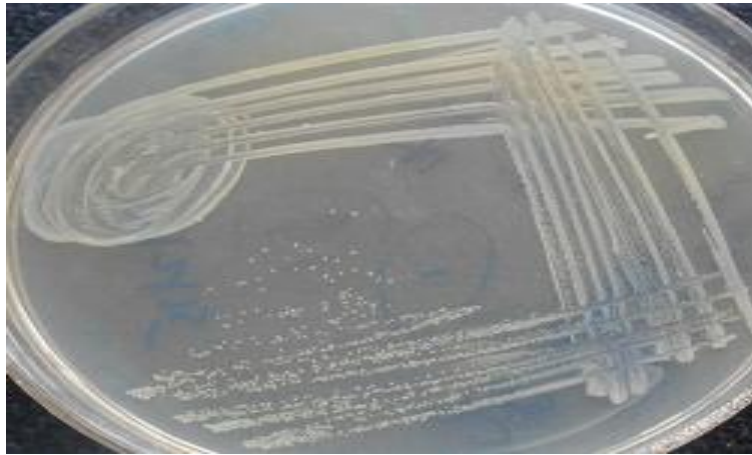


Fig.3 Gram staining image of CMGS-1

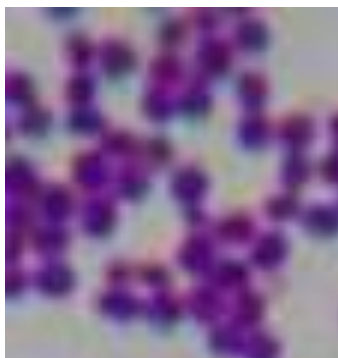


Fig.4 Optimization of decolorisation in RR-11 as sole carbon source

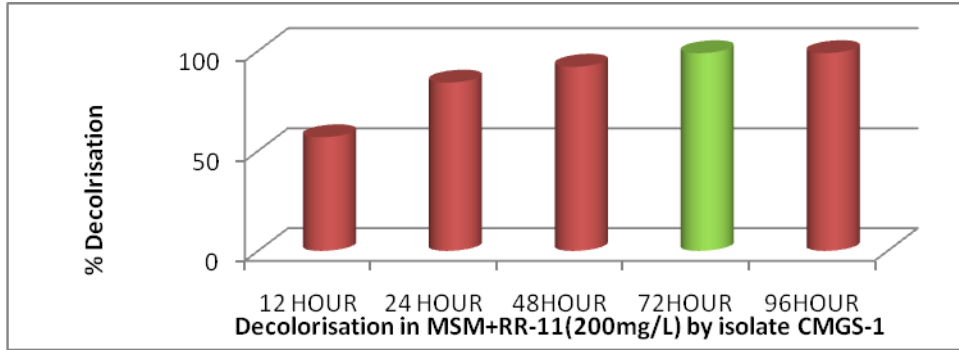


Fig.5 Optimization of decolorisation in RR-11 with 0.1% of yeast extract concentration

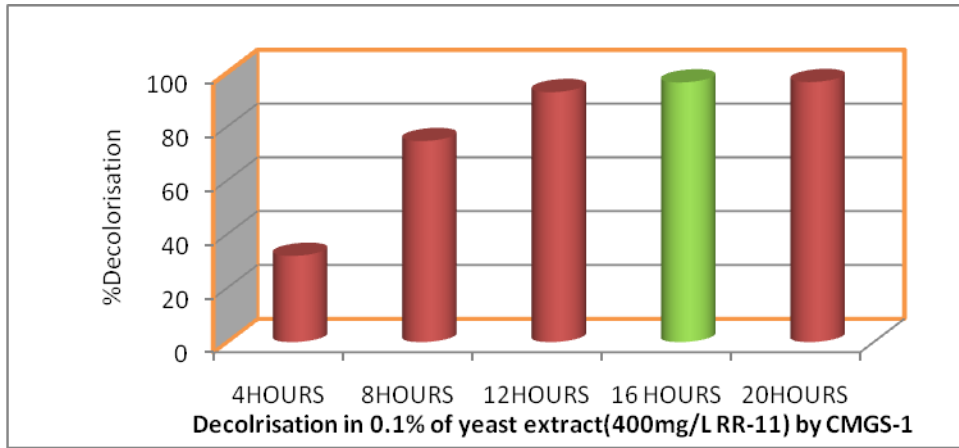


Fig.6 effect of static and shaking for maximum decolorisation by *Enterococcus casseliflavus* strain CMGS1

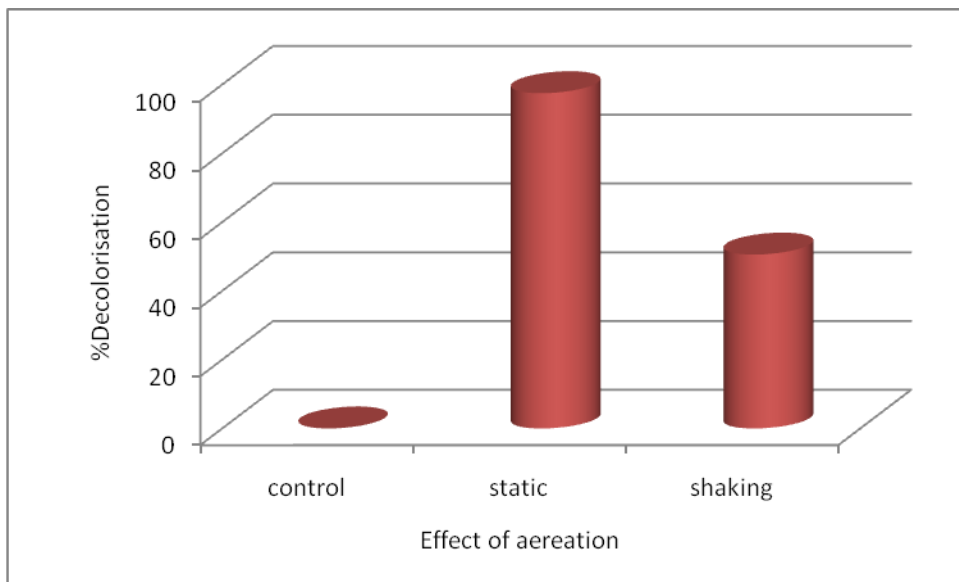


Fig.7 optimization of pH parameter for maximum decolorisation by *Enterococcus casseliflavus* strain CMGS1

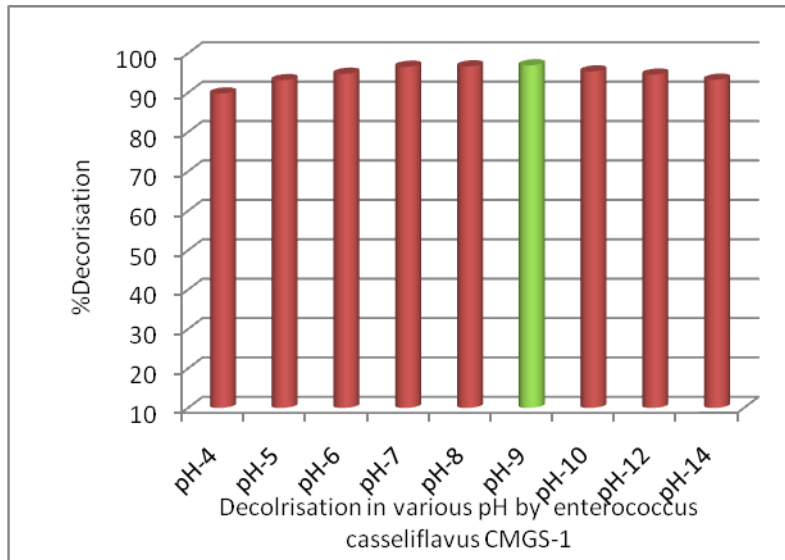


Fig.8 optimization of temperature for maximum decolorisation by *Enterococcus casseliflavus* strain CMGS-1

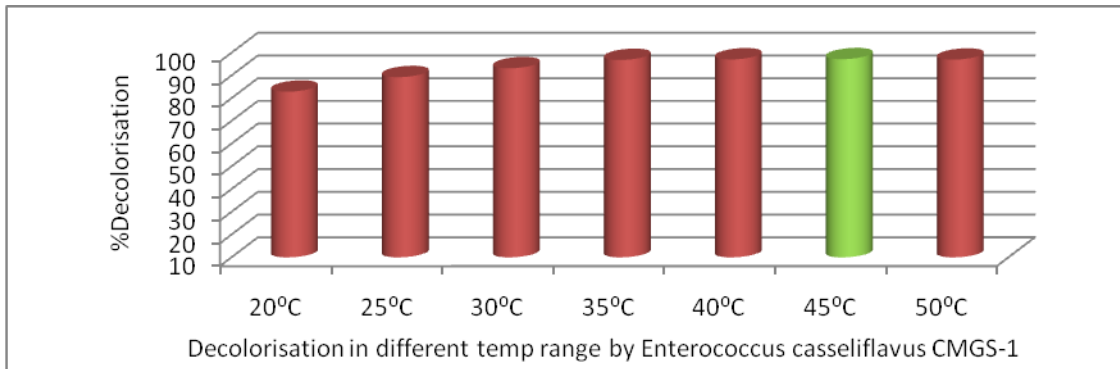


Fig.9 optimization of nitrogen sources for maximum decolorisation by *Enterococcus casseliflavus* strain CMGS1

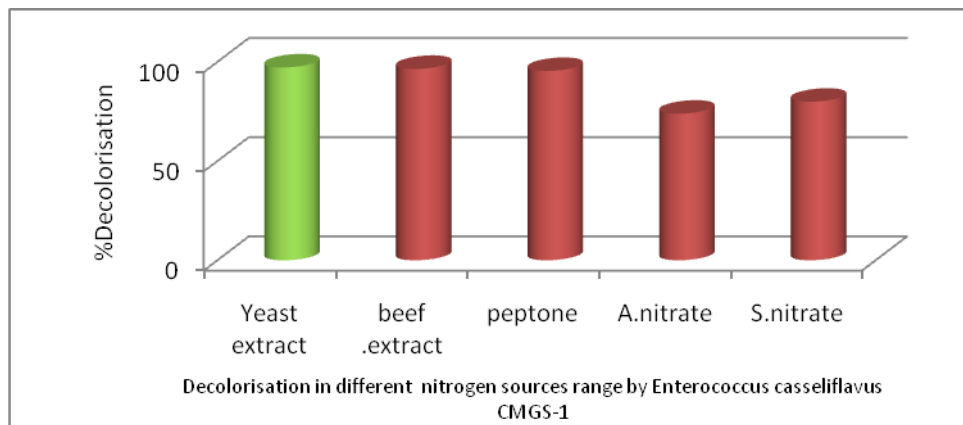


Fig.10 optimization of inoculum size for maximum decolorisation by *Enterococcus casseliflavus* strain CMGS1

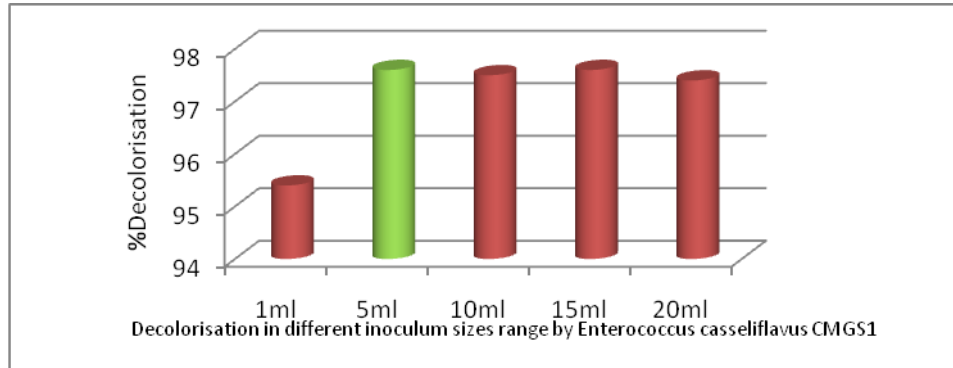


Fig.11 effect of yeast extract for maximum decolorisation by *Enterococcus casseliflavus* strain CMGS1

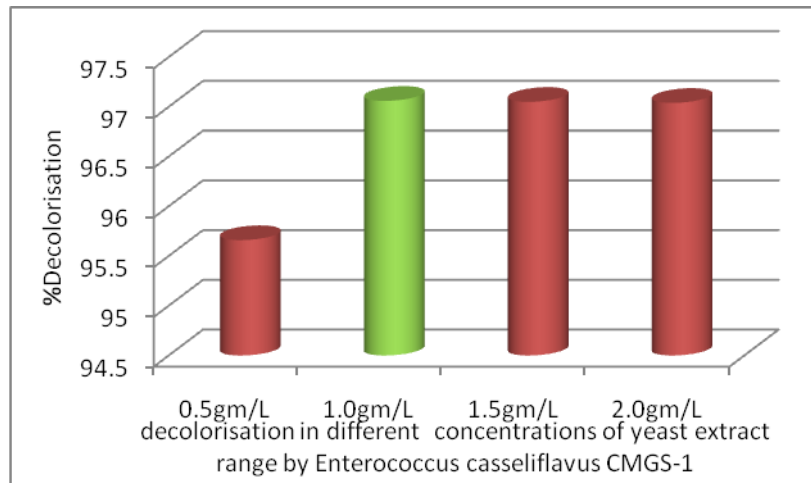


Fig.12 decolorisation in various reactive dyes by *Enterococcus casseliflavus* strain CMGS1

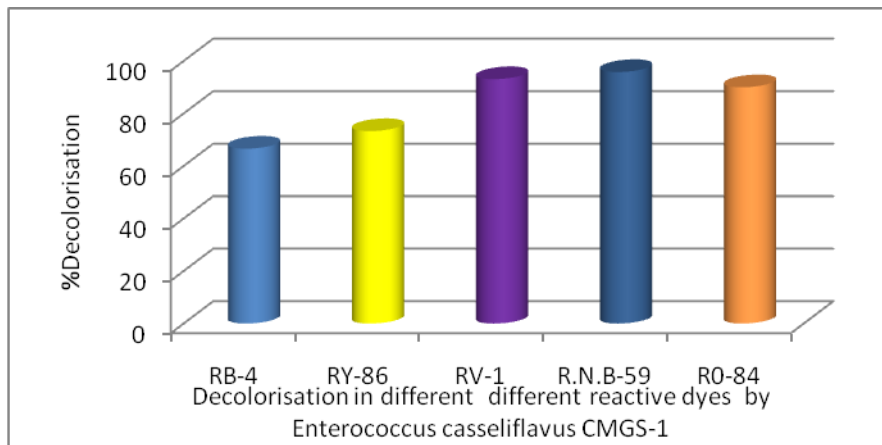


Fig.13 Control peak of reactive red-11

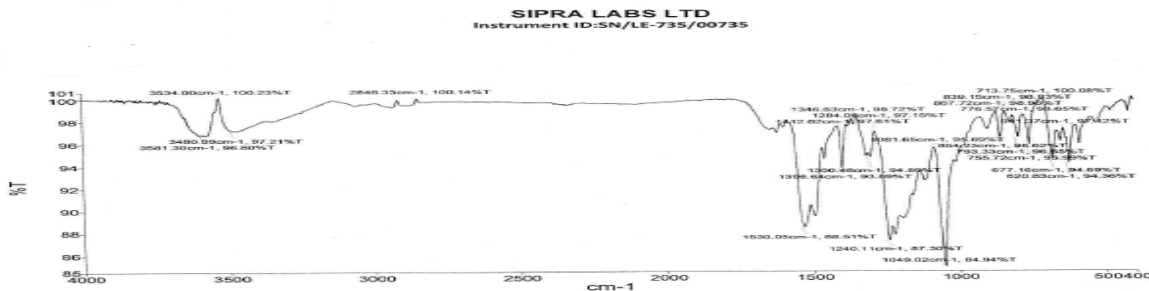
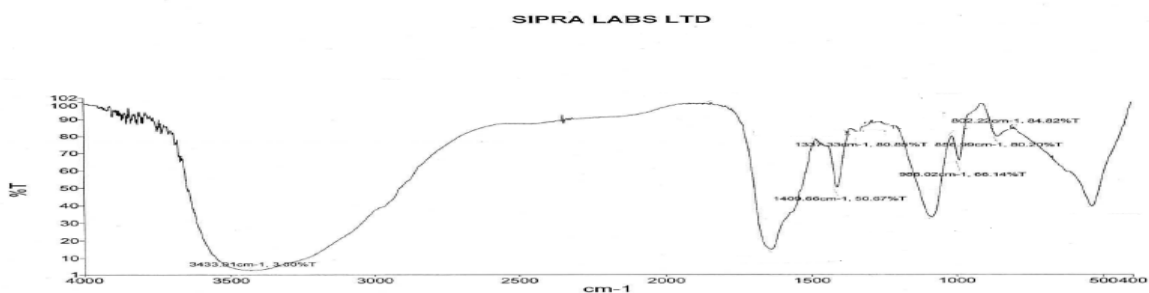


Fig.14 Degraded metabolite peaks of reactive red-11



Optimization of the inoculum concentrations

Inoculum concentration helps in the faster decolorisation, isolate CMGS-1 shown optimum inoculum size 5ml/100ML that is 97.06% of decolorisation. Inoculum size may depends on the organism report on Decolorization of Red 3BN by *B. cereus* optimum inoculum size was 8% and *B. megaterium* inoculums concentration was 10%. (Praveen Kumar *et al.*, 2012).

Optimization of various nitrogen sources

Nitrogen sources makes decolorisation in faster rate, various nitrogen sources were studies, and out of other nitrogen sources yeast extract helps in the effective decolorisation within shorter period of time. Similar with our results, *Lysinibacillus* RSV-1, strain, shown effective degradation up to 95% using yeast extract as a nitrogen source and not shown any degradation

using ammonium chloride, ammonium sulphate, urea etc., (Rajeshwari *et al.*, 2011).

Optimization of the yeast extract concentration: To get a highest decolorisation various concentration of yeast extract were selected, our isolate shown 1.0gm/L of yeast extract was optimum for the better decolorisation. (Kadpan *et al.*, 2000) stated that yeast extract act as dual source of carbon and nitrogen in decolorization of various dyes by bacteria.

Decolorisation of different reactive dyes by *Enterococcus casseliflavus* strain CMGS-1

Various reactive dyes were selected for the study of decolorisation and isolate CMGS-1 shown better results in all dyes, they were in reactive blue-4: it was 66.6. in reactive yellow-86-it was 73.3, in reactive violet-1 it was:-93.3%, in reactive navy blue -59 it

was 95.8, and in reactive orange-84: it was 90.0% of decolorisation was studied. Through these results isolate CMGS-1 having wide range of dye degrading capacity.

Biodegradation study by the FT-IR analysis:- Biodegradation Studies By FT-IR

Control reactive red-11, 23 different peaks were observed, spectroscopy run from 400 to 4000 cm^{-1} wavelength. The control spectrum of reactive red -11 was found 3581.30 cm^{-1} , 3535.00 cm^{-1} , 3480.99 cm^{-1} showing the intermolecular hydrogen bonding, and -OH Aromatic, O-H Stretching. 2848.33 cm^{-1} peak for N-H stretching amines. 1530.05 cm^{-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 peaks observed at- 802.22 cm^{-1} , 858.99 cm^{-1} , 980.99 cm^{-1} , 1337.33 cm^{-1} , 1409.66 cm^{-1} , 3433.91 cm^{-1} study shown complete degradation of reactive red -11, (Wojnárovits *et al.*, 2005) (Yingling *et al.*, 2008; Rajeshwari *et al.*, 2011; Lambert *et al.*, 1998).

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How to cite this article:

Madhuri R. Basutkar, Channappa T. Shivannavar and Subhaschandra M. Gaddad. 2016. Biodegradation of Reactive Red-11 by the Isolate *Enterococcus casseliflavus* CMGS-1 Strain. *Int.J.Curr.Microbiol.App.Sci*. 5(11): 565-578. doi: <http://dx.doi.org/10.20546/ijcmas.2016.511.067>