IJPSR (2017), Vol. 8, Issue 5 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 10 October, 2016; received in revised form, 06 January, 2017; accepted, 14 January, 2017; published 01 May, 2017

MYCOREMEDIATION OF TEXTILE DYES USING *TALAROMYCES FUNICULOSUM* JAMS1

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Keywords:

Fungus, FTIR, RSM, laccase

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ABSTRACT: The present study was planned with the aim to remove various dye decolorization using fungal strains. The fungi was isolated from soil samples collected from waste disposal areas and agricultural fields. Nine isolates were initially selected for testing their decolorization potential in the liquid medium. Three most effective strains were used to study the decolorization of four different dyes i.e Reactive Red, Navy Blue HER, Reactive Magenta B and Orange 3R. The strain belonging to *Talaromyces funiculosum* (M2F) was found to be the most potent strain. More than 70% decolorization was observed under optimal incubation conditions of temperature and pH. Overall, the dye decolorization was maximum at 250 mg/L dye concentrations at pH 5-6 under continuous shaking conditions. The culture conditions like pH, temperature, inoculum size and NaCl concentrations were also found effective in decolorization efficacy of the fungal strain which was studied using Response surface methodology (RSM) with Minitab 16 software. Qualitative and quantitative assays for determining the production of lignolytic enzymes such as laccases and peroxidises by the fungal strains were studied and a comparison of degraded and undegraded forms of the dye were done by using analytical techniques like FTIR, UV Vis spectroscopy and GCMS.

INTRODUCTION: Dyes make up an abundant class of organic compounds characterized by the presence of unsaturated groups (chromophores) such as -C=C-, -N=N- and -C=N-, which are responsible for the dye colors, and of functional groups responsible for their fixation to fibres, for example, $-NH_2$, -OH, -COOH and $-SO_3H$[1]. An estimate of more than 100,000 different synthetic dyes are available in the market of which about 700,000 tons are produced all over the world annually.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(5).2082-89

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (5).2082-89

These dyes find their uses in the textile, paper, cosmetics, food and pharmaceutical industries. Some of them are dangerous to living organisms due to their possible toxicity and carcinogenicity. About 10% is lost in wastewater and the soil surrounding the areas of disposal, which justifies the concern about the environment.

The development of treatment technologies suitable for the removal of color and reduction of toxicity of effluents containing these dyes are important. The development of decolorization technologies depend on available scientific knowledge. Based on the technical and economic needs, several new technologies involving the use of different physical, chemical and biological agents are being proposed and tested in various stages of commercialisation.

However, the latest studies have compiled the use of various microorganisms that are found to have the ability to degrade and detoxify the effect of such dyes at different levels. The bioremediation technology offers several advantages as it can be performed on site; generally, has lower cost and minimum inconvenience in the process and thus, eliminates the waste permanently. They can be used in conjunction with methods of physical and chemical treatments; has minimal environmental impact and, therefore, has good public acceptance, with the regulatory encouragement [2, 3].

Bioremediation of dyes in waste effluents by fungi is an alternative to conventional methods and a very promising area of study because of the relatively low expense involved. It has been shown that microorganisms are able to utilize a wide of variety of organic compounds as precursors for the synthesis of their own cell material or as sources of energy, even when such compounds are present at low concentrations in the environment. Various bioremediation studies have been done on fungi and a notable amount of literature can be found that indicates their extent of use in removal, and decolorization of dye (Mycoremediation). The key to mycoremediation is determining the appropriate fungal species to target a specific pollutant.

The efficiency of fungi in the process of decolorization is mainly based on the enzyme system that enables the degradation of various toxic compounds, including those that contain aromatic amines in its structure. Furthermore, the method has generally gained public acceptance, which makes the treatment using fungi a promising alternative to replace or complement the conventional treatments [4]. However, more studies are needed on the ability of fungi regarding decolorization and detoxification of dyes used by the textile industry. Many works relate the potential for decolorization of fungi from already affected areas [5, 6]. However, results obtained with fungi from unaffected areas have also shown to be promising [7, 8].

The present investigation utilises the use of 9 strains isolated from two field samples collected from waste disposal areas and agricultural soil, which were studied for dye degradation and tested for results.

MATERIALS AND METHODS:

Synthetic textile dyes: Four different types of synthetic textile dyes were used i.e., Methyl Red, Orange 3R, Reactive Magenta HB and Navy Blue HER. Synthetic textile dyes were used to study the efficacy of degradation by the fungal strains as these are the most commonly employed dyes used in textile industries and hence are more commonly found in the industrial effluents released during their manufacturing process.

Isolation of Microorganisms: The organisms were isolated from different areas collected from waste disposal areas and agricultural farm areas in Vellore, Tamilnadu, India. The soil samples (1gm each) were mixed in 10 ml of distilled water to prepare samples for serial dilution. Serial dilutions were prepared to dilutions of 10^{-3} and 10^{-4} and spread Plate technique was carried out on Potato dextrose agar (PDA) and Sabaroud dextrose agar (SDA) plates to isolate different fungal colonies. Triplicates were made and the inoculum was spread on PDA plates and incubated at room temperature for a period of 5-7 days which resulted in elevated cotton like growth. The subculturing was done in PDA and the plates were stored at 4 °C for further use. The dye degradation studies were done on potato dextrose broth media as maximum growth was found in PDB compared to SDB. Studies to detect the production of lignolytic enzymes by the fungal strains such as laccases, manganese peroxidases and lignin peroxidases were done initially on PDA plates supplemented with ABTS substrate [9] and further studies were done in liquid media to estimate enzymatic activity of the enzymes involved in degradation.

Screening for dye degrading fungal isolates: The colonies isolated from the mixed cultures were then tested for dye degrading potential of the fungal strain. Triplicates of each strain were made on potato dextrose agar medium containing 0.025% of each dye (Red, Magenta, Blue and Orange) respectively and then were inoculated onto the plate with a loop full of the fungal spores separately for each isolate [10]. The plates were then incubated at room temperature for seven days to allow the fungal colonies to grow and degrade the dye. Decolourisation of the inoculated plates with respect to the control plate was taken as the standard to identify decolourisation activity

exhibited by the 9 different fungal strains. The isolates which were able to decolorize High concentration was chosen for further studies including characterization by performing morphological assay, fungal biochemical tests for growth (study of utilisation of different carbon sources, nitrogen sources, amino acid assimilation and lipolytic activity) and 16s rRNA sequencing to identify the strain of fungus.

Identification of the potent isolate: The most potent fungal strain was primarily identified by Lactophenol Cotton Blue (LPCB) staining technique for morphological characteristics of the fungal cells. Further final identification was done with 18S rRNA gene sequence analysis. The genomic DNA of the fungus was isolated using AMpurE Fungal gDNA Mini Kit and amplified using polymerase chain reaction (PCR) technique with universal primers ITS1 and ITS4 [11]. PCR reaction mix of 50 µl final volume contained: 50 ng sample gDNA, 100 ng forward primer, 100 ng reverse primer, 2 µl dNTP's mixture (10 mM), 5 µl 10X Taq polymerase buffer, 3 U Taq polymerase enzyme and PCR grade water to make up the volume. Amplified PCR product was sequenced by using ABI3730xl genetic analyzer (Amnion Biosciences Pvt. Ltd. Bangalore, India). The sequencing result was submitted to the GenBank National Center for Biotechnology Information (NCBI) database.

Minimum Inhibitory Concentration (MIC) of fungal strain: The minimum inhibitory concentration (MIC) of the fungal isolate was checked for all the four dyes (red, magenta, blue and orange) in suspension cultures. Seed medium was prepared by inoculating a loopfull of mycelia into potato dextrose broth and incubating it for 24-48 h at room temperature. This seed medium was used as a culture suspension by inoculating into broth containing different concentrations (250mg/l, 500mg/l and 750mg/l) of each dye ie red, blue, magenta and orange individually. The culture flasks were incubated for 5-7 days and each day dye decolorization was observed manually as well as with the help of UV-Vis spectrophotometer. The assay was done to estimate the maximum dye concentration which can be tolerated by the isolate with luxuriant growth.

Enzymatic Activity Assay: In our present work, a relationship study was done to attribute the dye decolorization by the fungus to be enabled by the activity of the lignolytic enzyme, laccase being produced by the strain exhibiting maximum decolorization capability of all the four dyes.

Screening for Laccase Activity: Laccase activity of fungal strains is observed by decomposition of ABTS (2, 2'azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid) [12]. The fungal strains were tested for laccase production by growing them on LME Basal Medium agar plates containing 0.1% (w/v) of ABTS as a substrate. The LME basal medium (LBM) [(g /1 in distilled water)] consists of KH_2PO_4 (1), yeast extract (0.01), $C_4HI_2N_2O_6$ (0.5), $CuSO_4.5H_2O$ (0.001), $MgSO_4 \cdot 7H_2O$ (0.5), $Fe_2(SO_4)_3$ (0.001), $CaCl_2 \cdot 2H_2O$ (0.01) and $MnSO_4.H_2O$ (0.001). This colourless agar medium turns green due to the oxidation of ABTS to ABTS-azine in the presence of laccase.

Estimation of laccase activity: After the confirmation of laccase production by the fungal isolate, studies were done to estimate the enzymatic activity of the crude extract of enzyme produced during dye degradation by the organism. For which the fungal isolates were point inoculated into potato dextrose broth medium supplemented with the dyes and were allowed to grow by incubating at room temperature for 7 to 10 days. The inoculums from the new culture flasks were then subjected to undergo reaction with ABTS substrate at 3 day intervals and the cultures were centrifuged at 10,000g for 30 min at 4 °C to filter out the supernatant through WhatmanNo.1 filter paper. The reaction mix consisted of 0.5 ml of ABTS (0.45mM) with 1.2 ml phosphate buffer of pH 6 (0.1mM) and 0.5ml of our crude enzyme sample filtered out after centrifugation. The reaction mixture was then incubated at 27 °C for 10 minutes and the absorbance values were recorded at 420 nm. The oxidation of the ABTS present as substrate by the crude enzyme produced by the degraded fungal dye sample can be observed by an increase in the absorbance values. The absorbance values were recorded for a period of 10 days. The three strains producing positive result to the laccase assay were used [13].

The absorbance values for a period of 10 days were recorded. The activity of the crude enzyme extract was estimated using the formula:

$$LA\left(\frac{U}{L}\right) = \frac{\Delta Abs}{\Delta t \varepsilon l} * \frac{\text{total Assay Volume(2.2 ml)}}{\text{Enzyme sample Volume(0.5ml)}}$$

where, ΔAbs=absorbance at 420nm
Δt = time taken for the reaction(10 minutes)
E = extension coefficient of ABTS (3.6 *10^4 $M^{-1}cm^{-1}$)
l=diameter of the cuvette (1 cm)

Response Surface Methodology (RSM): Four important parameters like pH (X1), NaCl conc. (X2), Temperature(X3) and inoculum size (X4) were chosen as the independent variables and percentage decolorization was the dependent response variable. Each of the independent variables was studied at five different levels as per CCD in four variables with a total of 31 experiments. Percentage dye decolorization corresponding to combined effects of four components was studied in their specified ranges, NaCl concentration: 0.5–2.0% (w/v), pH: 4 – 9, Temperature: (25 °C to 35 °C) and inoculum size: 1 to 5 % (v/v). The other two process variables pH and temperature were kept constant at 6 and 35 °C respectively throughout the 31 experiments.

FTIR Spectroscopic studies: Fourier Transform Infrared Spectroscopy was done to find out the different infrared spectrum of the sample being analysed. The sample for FTIR analysis was prepared by separating the biomass component from the fungal dye cultures and supernatant was taken after centrifugation for FTIR analysis. Infrared (IR) spectra of the parent compound and the sample after degradation with fungal strain recorded in the frequency range of 4,000-400 cm^{-1} with a Fourier transform infrared (FTIR) spectrophotometer (8400 Shimadzu, Japan, with Hyper IR-1.7 software for Windows) with a helium-neon laser lamp as a source of IR radiation. Pressed pellets were prepared by grinding the extracted samples with potassium bromide in a mortar with 1:100 ratio and analyzed in the region of 4,000-400 cm^{-1} at a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION:

Isolation, Screening and Identification of Fungal Strains: A total of 9 fungal strains were isolated from the two soil samples. Three different types of fungal strains were isolated from the soil sample collected from agricultural fields and six different types of fungal strains were obtained from the soil sample collected from waste disposal areas. The isolates were pure cultured onto PDA plates and stored at 4 °C for further experimental procedures.

Screening for potent fungal strain: The 9 different strains of fungal isolates were tested for dye decolourisation by point inoculation on potato dextrose agar plates containing 0.025 g of each dye (Methyl Red, Orange 3R, Navy Blue HER and Reactive Magenta HB). **Table 1** indicates the fungal decolorization by all the 9 fungal strains.

TABLE 1: DYE DECOLORIZATION BY SOME FUNGAL STRAINS

Sl no.	Sample	Navy Blue HER	Orange 3R	Methyl Red	Reactive Magenta HB
1.	M1C	++	--	++	+
2.	M2B	++	+	+	+
3.	M2D	++	--	+	++
4.	M2E	+	-	+	++
5.	M2F	+++	+	++	+++

Identification of dye degrading strain: Lactophenol cotton blue staining was done to identify the fungal strains and to observe various fungal appendages like conidia, hyphae and mycelia via a microscope to study the fungal characteristics. The staining showed that the cultures belong to *Penicillium* sp. Further, the BLAST result of the 18S rRNA gene sequence analysis revealed the close relationship with 99% similarity to the sequence of *Talaromyces funiculosum.* Multiple sequence alignment and phylogenetic tree is given in **Fig. 1**.

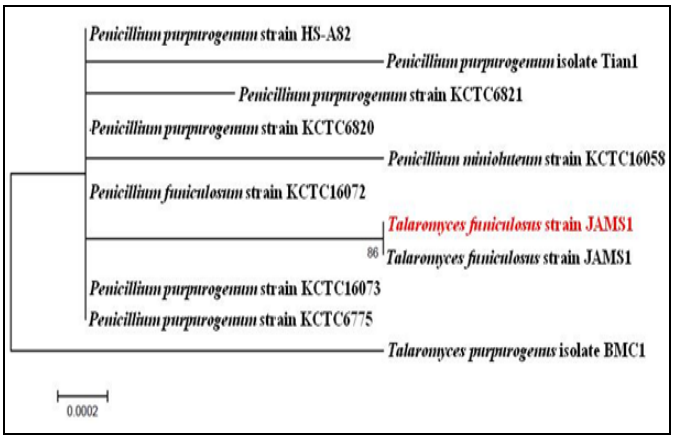


FIG. 1: PHYLOGENETIC TREE OF STRAIN JAMS1

Laccase activity: The Laccase production by the fungal strain was observed by ABTS assay. ABTS (0.015gms) was added to PDA medium and then incubated for a period of 4-5 d. This colourless agar medium turns green due to the oxidation of ABTS (2, 2'azino – bis (3-ethylbenz-thiazoline-6-sulfonic acid) to ABTS-azine in the presence of laccase. Three fungal strains showed positive results to laccase activity (M2F, M2C and M1C). Development of green colour in the media (as shown in **Fig. 2**) indicates production of laccase enzyme.

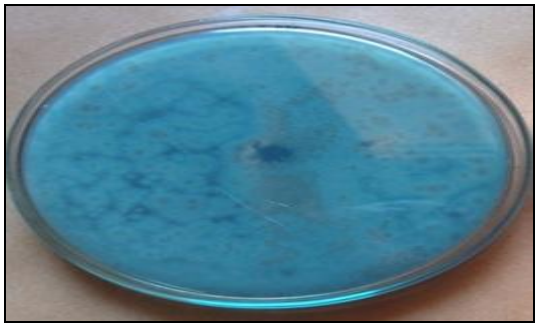


FIG. 2: LACCASE PRODUCTION FROM POTENT STRAIN JAMS1

Estimation of Laccase activity: The oxidation of the ABTS present as substrate by the crude enzyme produced by the degraded fungal dye sample can be observed by an increase in the absorbance values. The absorbance values were recorded for a period of 10 days. The three strains producing positive result to the laccase assay were used. The absorbance values for a period of 10 days were recorded. The specific activity of laccase was calculated with the extinction coefficient for ABTS as $1.6*10^4$ M^{-1} cm^{-1} and a plot of activity vs time was prepared (**Fig. 3**). The enzymatic activity of crude fungal laccase was found to be 209.84 units per ml and maximum activity was recorded on the 9^{th} day of incubation for *Talaromyces funiculosum.*

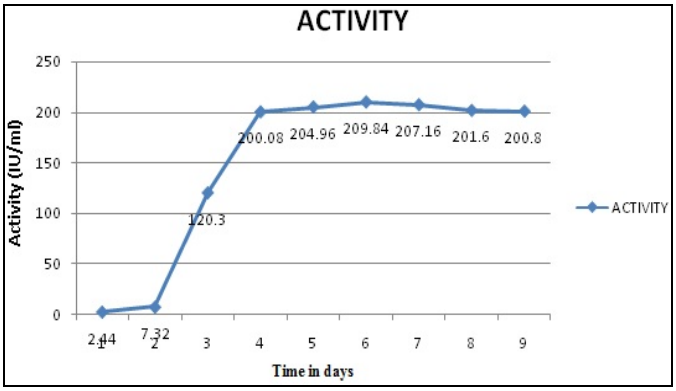
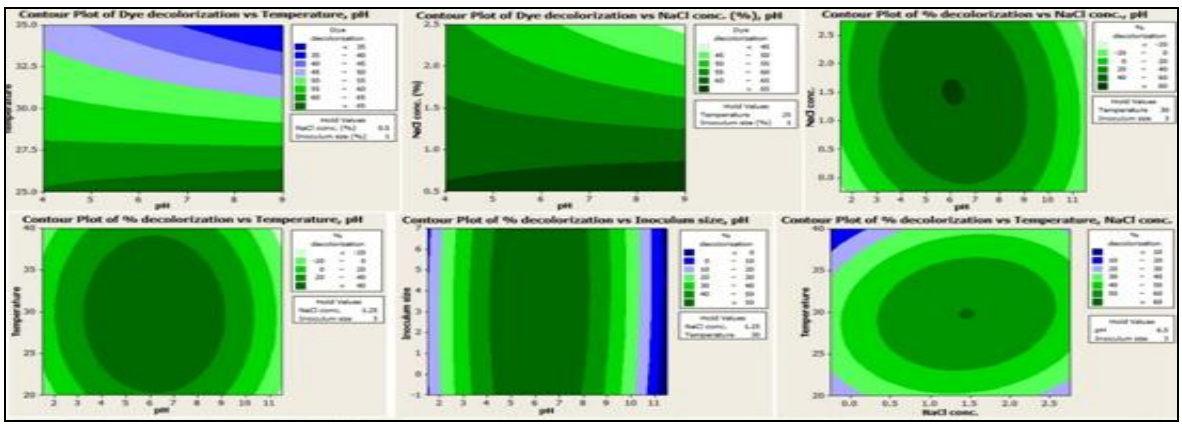


FIG. 3: ESTIMATION OF LACCASE ACTIVITY

Response Surface Methodology: It was observed that for the given dye at the particular concentration of 0.25% w/v of the culture medium, the most optimum values for decolorization were obtained at a pH of 5 to 6 and at temperature conditions of 25 ^{0}C to 35^0 C. Decolorization varied markedly with the conditions tested, in the range of 11–80%.

Contour plots: These plots are a graphical representation of three dimensional data on a two dimensional surface. They provide a two dimensional plot in two axis. The plot between percent decolorization against temperature and pH depicts maximum decolorization at hold values of NaCl concentration at 1.25 % (w/w) and inoculum size of 3 % (v/v) between pH ranges from 5 to 7 at temperature ranges of 25^{0}C to 35 ^{0}C. Similarly, the plot with NaCl concentration and pH depicts maximum decolorization at NaCl conc. of 1% to 2% (w/v) at pH ranges from 6 to 7, at hold values of temperature at 20 °C and inoculums size of 3% (v/v). Whereas with inoculums size and temperature the plot depicts shows maximum decolorization at temperature ranges between 25 °C to 35 °C and inoculums sizes ranging from 1% to 5% (v/v) (**Fig. 4**).



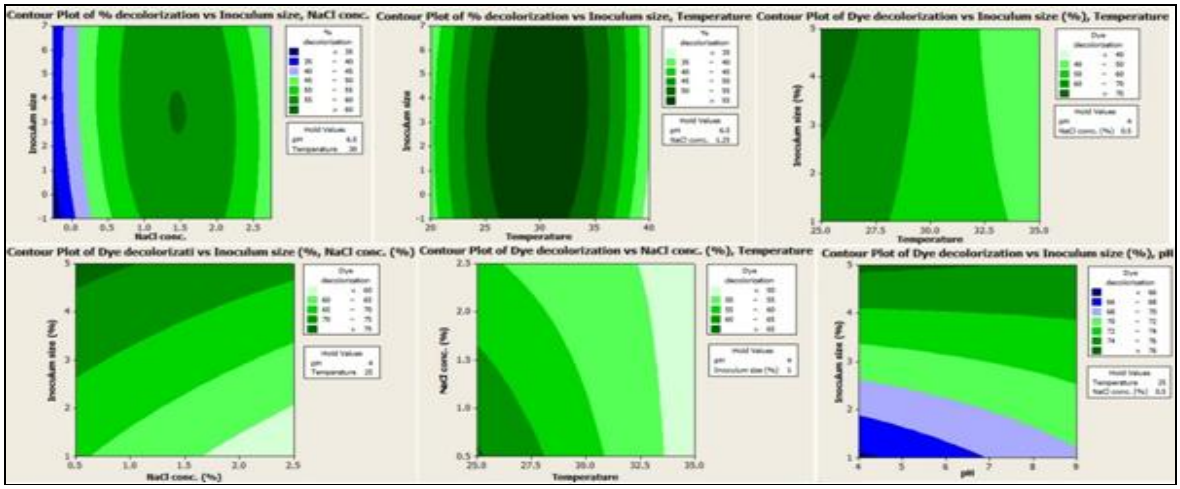


FIG. 4: CONTOUR GRAPHS SHOWING 2D PLOTS OF THE VARIABLES

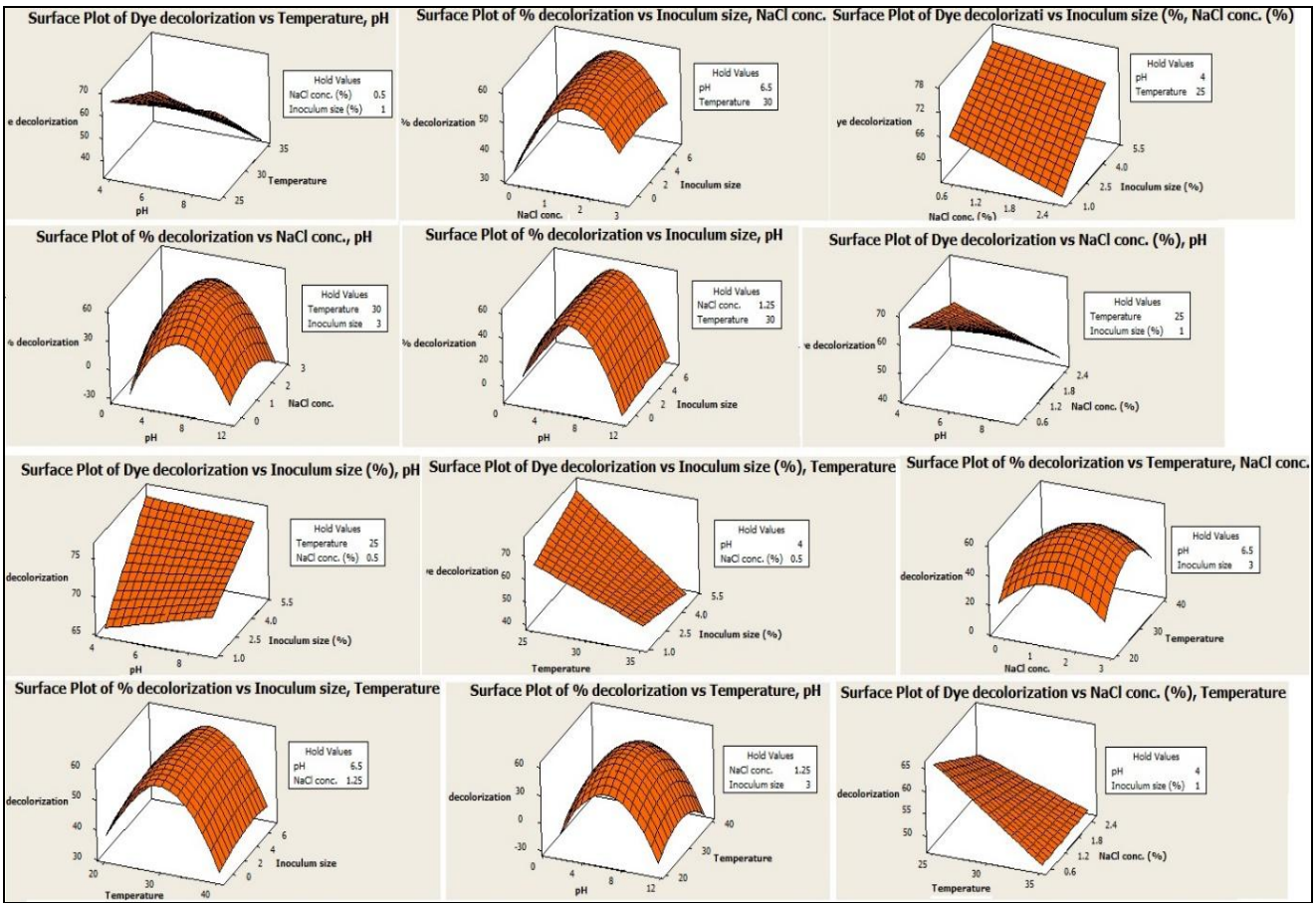


FIG. 5: SURFACE PLOTS GIVING 3D REPRESENTATION OF THE INTERACTIONS BETWEEN THE VARIABLES

Surface plots: The profiles of surface response generated from the experimental model in the optimization of two variables each. The surface plots show the 3D representation of the interactions between the variables with respect to the response which in this case is percent decolorization. Also the hold values are clearly visible in the graphs (**Fig. 5).**

FTIR Analysis: The FTIR data analysis of the samples (**Fig. 6**) gives the different peaks of the components present in the sample. The peaks are obtained as a result of absorbance of the rays in the IR spectra by the different components at different wavelengths. Each peak corresponds to a particular component present in the degraded dye samples.

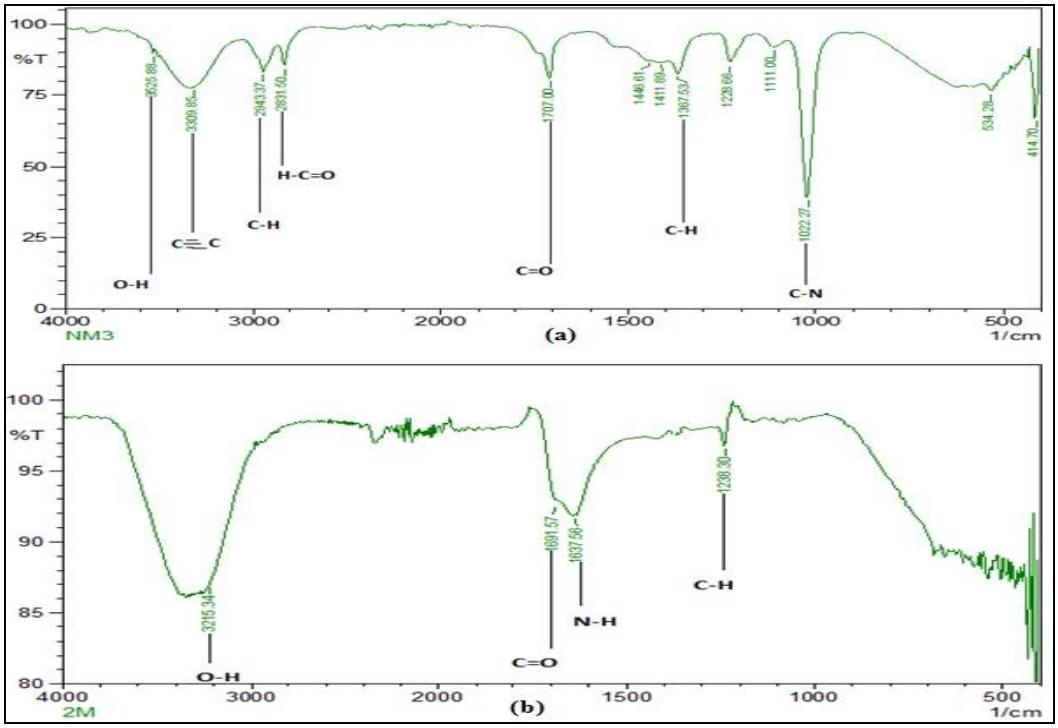


FIG. 6: FTIR SPECTRUM OF DEGRADED REACTIVE MAGENTA HB DYE

The various peaks indicate different functional groups in the degraded dye sample. In plot 14, frequencies in the range of 3200 cm^{-1} to 3500 cm^{-1} indicates an O-H bond, depicting the presence of alcohols and phenols as the functional groups. The peak values 1665cm^{-1} to 1710 cm^{-1} indicates the presence of α, β- unsaturated aldehydes and ketones. The presence of peak values between 1350cm^{-1} to 1370cm^{-1} represents the presence of primary amines. Further, the peak value at 1238 cm^{-1} indicates the presence of an aliphatic amine.

The FTIR spectrum of the undegraded reactive magenta HB dye basically shows seven significant peaks. The peak at 3625 cm^{-1} indicates the presence of an -OH bond indicating functional groups as alcohols or phenols. The peak values at 3309cm^{-1} and 2943 cm^{-1} indicates the presence of functional group alkynes and alkanes respectively. The peaks at 2430 cm^{-1} indicates aldehydic groups. The peak value at 1707 cm^{-1} indicates a carbonyl group and can be attributed to α, β-unsaturated aldehydes and ketones. The presence of -CH and -CN bonds indicate the peaks at 1367 cm^{-1} and 1022 cm^{-1}, which represent functional groups alkanes and aliphatic amines. After the dye decolorization, by comparing the FTIR spectra of degraded and undegraded dyes, we can see that the alkyne group in the undegraded dye is converted to alkane group in the degraded dye. Further it can be noticed that the no. of peaks in the undegraded dye has reduced to four peaks in the FTIR spectra of the degraded dye. This indicates that the number of reactive functional groups have been reduced after the dye decolorization reaction indicating the chemical reaction or change that occurs during the process.

CONCLUSION: The present study aimed to study and identify the most efficient fungal strain effectively degrading all the four dyes i.e., Reactive red, Navy blue HER, Orange 3R and Reactive Magenta HB at dye concentrations of 0.025% (w/v). The samples were isolated from the soil samples collected from industrial waste disposal areas and agricultural farms. Amongst the nine isolates, Isolates obtained, *Talaromyces funiculosum* (M2F) was found to be most efficient. However, the maximum degradation by the *Talaromyces funiculosum* was identified for the Reactive Magenta HB dye. The spectral studies on the degraded dye and the further comparison of the IR spectra of undegraded and degraded dye indicated degradation of alkynes and aldehydic functional groups to alkanes.

ACKNOWLEDGEMENT: The authors are greatly indebted to Vellore Institute of Technology, Vellore for their constant encouragement, help and

support for extending necessary facilities to successfully complete the experiment.

CONFLICTS OF INTEREST: There are no conflicts of interest to declare.

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How to cite this article:
Chatterjee A, Singh N and Abraham J: Mycoremediation of textile dyes using *Talaromyces funiculosum* JAMS1. Int J Pharm Sci Res 2017; 8(5): 2082-89.doi: 10.13040/IJPSR.0975-8232.8(5).2082-89.