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A STUDY ON DEGRADATION OF 2,6 DICHLORO INDOPHENOL USING *Fusarium* SPECIES

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ABSTRACT

Background: 2,6-dichloro indophenol is the most common textile dye used across the globe for dyeing textile fibers. The textile industries discharge untreated effluents and are let into city drains that enter rivers and lakes. This brings out changes in pH, Total Organic Carbon, Biological Oxygen Demand, Chemical Oxygen Demand, and other water quality parameters are affected. Several physical and chemical methods are employed for the removal of the contaminating dye from wastewater such as chemical precipitation, adsorption, reduction, sonication, electrochemical treatment, etc.

Methods: In the present study, 2,6-dichloro indophenol has been used for the dye degradation analysis using fungi isolated from the soil. The study focuses on the extent of decolorization of the dye at various concentrations by inoculating with fungi isolate *F. equiseti.* This work is to understand the effectiveness of *Fusarium equiseti* in the degradation of 2,6-dichloro indophenol. The decolorization of the dye was monitored by UV-Visible spectrophotometer analysis. The FTIR analysis further substantiated the degradation of 2,6-dichloro indophenol.

Results: After performing spectroscopic analysis on the 2nd, 4th, and 6th day of incubation, the dye showed a maximum of 75.23% degradation within 6 days with a dye concentration of 0.02%. UV-

Visible spectrophotometer analysis demonstrated an absorption peak at 500 – 550 nm for dye decreased gradually with the increase of exposure time indicating a degradation reaction. The presence of a weak band was observed at 3281 cm $^{-1}$ corresponding to O-H stretching alcohol and new medium bands were formed during the incubation suggesting the conversion of alcohol in the presence of *Fusarium equiseti* to alkane. The study of enzyme assay is to measure the Pectinase activity using Oswald's Viscometer, showing the reduction in the viscosity after 2 hours indicating the increase in the pectinase activity with the dye and fungal culture. The pH study shows maximum degradation at pH 4.

Keywords*:* Dye degradation, 2,6-dichloro indophenol, *Fusarium* sp, Decolorization, Bioremediation

INTRODUCTION

Environmental pollution due to rapid industrialization and urbanization has become a major problem in this century. Numerous pollutants produced by humans reached directly or indirectly to the aquatic systems. Textile dyes are chemical compounds having xenobiotic nature and are highly thermal and photostable which makes them refractory for biodegradation. These dyes are usually synthetic in nature and are derivatives of coal tar or petroleum-based intermediates. Dyes are marketed in the form of liquids, powders, granules, pastes, or liquid dispersions. Advances in the dyeing machinery have led to newer dyes, that are manufactured to meet the demand, which leads to serious environmental concerns **[1]**. High concentrations of the dyes in the water bodies affect their reoxygenation capacity, thus affecting adversely on the aquatic flora and, this, in turn, can affect man through the food chain **[2]**. The effluents even reduced the growth of the crop plants, along with which also decreases the carbohydrate, protein, and chlorophyll content of the plants indicating the toxic nature of the dyes **[3]**.

The dyes are organic compounds and exhibit a high level of solubility in the water which makes it extremely difficult to remove them from water bodies **[2]**. Bioremediation of textile dyes by transforming or mineralization the contaminants by using enzymes, plants, bacteria, fungi, and extremophiles is the main point of focus in present-day research. However, despite a few disadvantages, bioremediation is progressively enhanced using many biotechnological techniques **[4]**. This led to the use of many microorganisms which gives us a sustainable solution over conventional physicochemical treatments. It is also said to be economically cost-effective with the use of microorganisms and enzymes. The physicochemical methods involved in

dye removal or degradation are high costs due to electricity, inputs, or operation inconveniences generated by the sludge disposal **[5]**. In addition, bioabsorption of microorganisms using dead bacteria, fungi, and yeasts is used when dyes are very toxic through absorption, deposition, and ion exchange **[6]**. Microbial enzymes such as peroxidases and phenol oxidases are also used for the degradation of azo dyes. Independent peroxidase enzymes have shown a high affinity for dyes such as bromophenol blue, thymol blue, etc **[8].**

The dye degradation process by microorganisms involves three steps that are biosorption, bioaccumulation, and biodegradation when mediated by fungi. The primary mechanism of decolorization is the adsorption of the dye to the microbial cell surface. In fungal biomass, biosorption is a metabolically dependent process that involves the binding of dyes to fungi, this way decolorization can occur either by living or dead fungal biomass **[9].** These biomasses consist of chitin which is a natural polysaccharide and its derivates. The functional groups present on the cell wall of fungi play an important role in the degradation of dye along with the reduction in organic pollutants. The functional groups are composed of various carboxyl groups, amino groups, lipids, melanin, sulfates, and phosphates. Fungi can also be used for metal removals through adsorption, chemisorption, complexation, coordination, chelation, physical adsorption, and micro precipitation **[10]**.

White rot fungi have the unique ability to cleave carbon-carbon bonds in polycyclic aromatic hydrocarbons and are used for the mineralization of synthetic dyes **[11].** *Neurospora crassa* is widely used for the degradation of phenols and other dyes from wastewater. But the main disadvantage of using white-rot fungi is their long growth cycle and high nutrient requirement **[12].** *Fusarium* is a soil fungus and is associated with plants all around the world. These are cosmopolitan and occur in different ecosystems and colonize a variety of substrates **[13].** However, the diversity of *Fusarium* depends on various parameters which are poorly understood like soil characteristics, crops, cultural practices, and human activities **[14].** It belongs to a large genus of filamentous fungi, also called hyphomycetes. Most of the *Fusarium* species are harmless saprobes, however, few species produce mycotoxins in cereal crops affecting human and animal life **[15]**.

MATERIALS AND METHODS

Collection and isolation of pure cultures

Soil samples were collected from various locations at Kristu Jayanti College, Bengaluru on 15th February 2021 for the isolation of *Fusarium* species. Isolation of the fungi was done by serial dilution method and dilution 10^{-5} was used. The diluted samples were streaked onto Petri plates containing Potato Dextrose Agar (PDA) media, and the composition of PDA media was used **[16].** These plates were incubated for 72 hours at 28 °C. This was done to ensure that the isolates were axenic, and the isolates were sub-cultured on fresh PDA plates several times. Colonies with distinct characteristics were selected and purified by repeated streaking on PDA plates, and the pure cultures were stored at 4°C until needed. Different types of fungal colonies were observed on the medium, pure colonies were isolated and cultures were stored on PDA (potato dextrose agar) plates. Mycelial tip fragments were placed on a slide and stained with cotton blue stain and observed under a light microscope (Olympus) with 10X, 40X, and 100X oil immersion magnifying lenses.

Primary screening of the Fungal Strain:

Identification of the soil fungal strain was done previously based on their morphological characters **[17]**. The morphological characterization of the fungal strain on the PDA plates was carried out by visual observation, and the microscopic identification was done using a lactophenol cotton blue stain and observed under the microscope.

Molecular Identification of the Fungal Strain:

DNA extraction, amplification, and sequencing were performed as described by Gilgado *et al*., (2005) with some modifications **[18]**. The fungal strain identification was done by performing 28S rRNA Sequencing. The complete genome identification was done using the Bioinformatics tool BLAST.

DNA extraction from isolated *Fusarium* that were morphologically identified cultures was inoculated in 150 ml of 2% PDB (Potato Dextrose Broth) and incubated for 15 days at 25 ± 2 °C. Around 1g of the mycelial mat was minced with sterilized sand in a mortar and pestle. The homogenized mixture was re-suspended in 10 ml of extraction buffer. Extraction buffer consists of 1% CTAB (Cetyl trimethylammonium bromide), 0.7 M NaCl (sodium chloride), 25 mM EDTA (Ethylenediaminetetraacetic acid), 50 mM Tris HCl, and 1% ß-mercaptoethanol. The samples were incubated at 65 °C for 1 hr. The homogenized mixture of samples was subjected to phenol-chloroform-isoamyl alcohol (25:24:1) for extraction of DNA and centrifuged the samples for 10 min. The upper

phase was mixed with 0.6 volumes of isopropanol for the precipitation of DNA and incubated at -20 °C overnight. After decanting the supernatant, DNA pellets were observed and rinsed with 200 µl 70% ethanol. Pellets were air-dried and sterile distilled water (pH 7.2) was added to dissolve the pellets completely and stored at -20°C **[19]**.

Decolorization studies

Dye degrading potential was determined by inoculating the fungal isolate in a 250 ml Erlenmeyer flask containing 100 ml of mineral salt medium **[20]**. Decolorization studies were carried out by dye treatment at the different concentrations of 0.01%, 0.02%, and 0.03% along with a control (without inoculum). The fungal strain was inoculated into each test tube except the control. The test tubes were kept undisturbed at room temperature (25 °C) for 10 days. Decolorization of the dye was monitored at regular intervals using the colorimeter every 48 hours. The change in the OD values indicated the extent of decolorization at various concentrations. The dye decolorization by the fungal strain was calculated using the formula:

$$\text{Percent Decolourization} = \frac{(AI - AF) \times 100}{AI}$$

Where, AI – is the initial absorbance and AF – is the final absorbance **[21]**.

UV -Visible spectrophotometer analysis:

At intervals of 4 hours, the sample was drawn from each flask and analyzed for concentration of dye. The sample was centrifuged at 10000 rpm for 10 mins and the supernatant was measured at 540 nm using a UV-Visible spectrophotometer. The degree of dye degradation during incubation period is measured by the decrease in optical density. The optical density of the decolorized dye was observed at different wavelengths ranging between 200 nm – 1000 nm using the spectrophotometer. Three different concentrations (0.02%, 0.03%, 0.04%) of the dye were used for measuring the optical density. These dye concentrations were added into the test tubes containing SDA broth along with the fungal strain, and the tubes were kept at room temperature (25 °C) for 4 days. On the 4^{th} day, the UV -Vis Analysis was performed **[22]**.

FTIR analysis:

FTIR analysis of dye before and after decolorization was performed to observe changes in structure and for detailed information. The FTIR analysis was performed in the mid-Infrared region of 400 – 4000/cm. Before the analysis, the sample was mixed with pure KBr in the ratio of 5: 95, and pellets were then fixed in the holder for analysis **[23]**. The FTIR spectra provide

information on the molecular structure of the dye after it has decolorized. For the FTIR Analysis, 0.02% concentration of the dye samples from day 4 and day 8 along with the control were dried in a hot air oven and 0.1g of the sample was used for the FTIR Analysis.

Evaluation of pathogenicity of the isolated strains of *Fusarium* for pectinolytic activity

Czapek's medium was prepared without sucrose but containing 3% pectin to detect the pectinolytic activity. Czapek's medium with sucrose served as a control. The 50 ml media was dispensed in 250 ml Erlenmeyer flasks, sterilized, and inoculated with an 8 mm culture disc of Fusarium isolated from five different locations. Triplicates were maintained for pectin-containing media. After 7 days of incubation at room temperature (28 ± 2 ° C), the mycelium was removed by centrifugation. The culture filtrates were assayed for pectinase activity by determining the changes in the viscosity of pectin. The reaction mixture consists of 4 ml of 1% pectin dissolved in boric acid buffer at pH 8.6, 1 ml of tris-acetate buffer, and 2 ml of culture filtrate. The mixture was transferred to an Oswald-Fenste Viscometer and loss in viscosity was determined after 2 hours of incubation **[10]**. One unit of pectinase activity corresponds to activity resulting in a 50% decrease in relative viscosity of 1% pectin solution for a duration of 5 minutes and appropriate pH **[24]**.

Effect of pH on the decolorization of the dye:

To confirm the decolorization of the dye is not due to the change in the pH of the medium, and adsorption or absorption by the change in the pH of the culture filtrate with the addition of HCl or NaOH. The effect of pH on the decolorization of the dye was studied by varying pH from 4 to 9 with the increment of 1 pH unit. Czapek's medium inoculated with culture disc of Fusarium was incubated at room temperature (28 ± 2 ° C), static condition for a duration of 4 days. The experiment was performed at 100 mg/ml of dye concentration **[25]**.

RESULTS AND DISCUSSION

Isolation and identification of fungal strain

Potato Dextrose Agar (PDA) media showed growth for fungal species, from which *Fusarium sp* were identified and re-streaked to maintain the pure culture for further use. Morphological and microscopic identification was done, During the morphological identification, it was observed that there was white cottony growth, when observed under the microscope, based on their microscopic characteristics the fungal strain when observed under the microscope at 40X, appeared to be sickle-shaped which confirms

that the fungal strain is *Fusarium equiseti.* Macroscopic characters for each isolate on PDA (**Figure 1)**. The isolate was cultivated on PDA and prominent mycelia appeared within 3 days. Mycelia were characterized as off-white, fairly dense, and showing yellow pigmentation at the periphery. The growth pattern of mycelia was in thick concentric rings **[26]**.

Mycelial tip fragments were stained with cotton blue stain and observed under a light microscope (Olympus) with 40X **(Figure 2**). To confirm the morphological and microscopic identification of the *Fusarium sp*, molecular identification was done by outsourcing using the 28S rRNA Sequencing method, bioinformatics tool BLAST was used to confirm the *Fusarium* species, which was found to be *Fusarium equiseti*. The genetic identification of the fungal isolate was accomplished by the 28S rRNA sequencing of the DNA extracted (**Figure 3**). The molecular identification was based on sequencing analysis for isolate *F. equiseti* with similarity percentages of 98.5% and 99.5% (**Figure 4**).

Figure 1: Pure culture of *Fusarium equiseti* on PDA plate

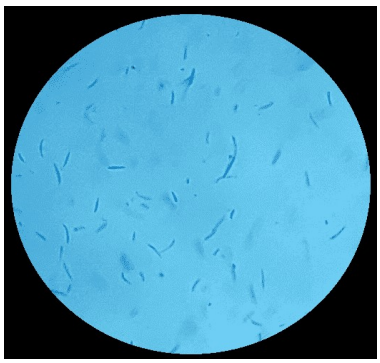

Figure 2: Microscopic Observation of *Fusarium equiseti* using Lactophenol cotton blue under 40X

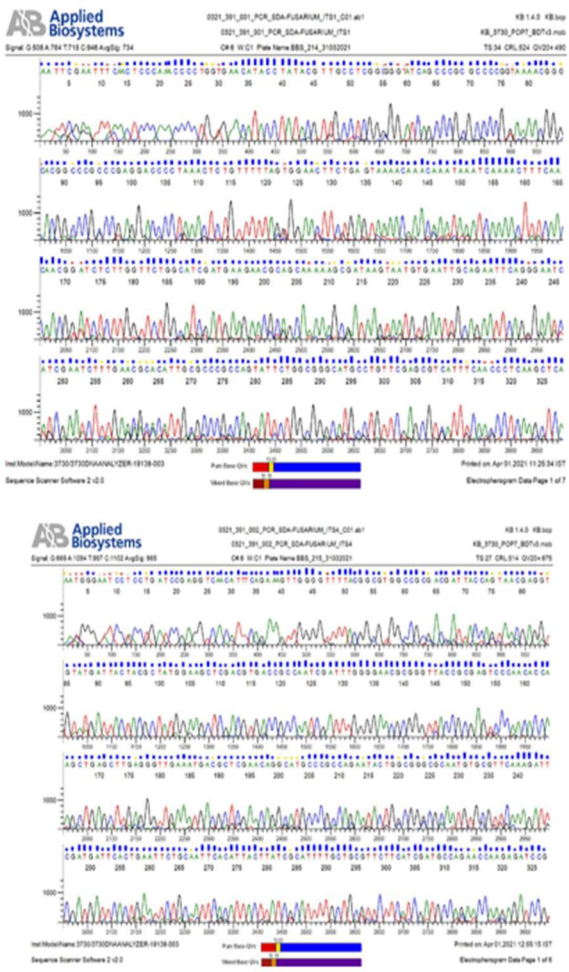

Figure 3: Chromatogram of 28S rRNA sequence of *Fusarium equiseti*

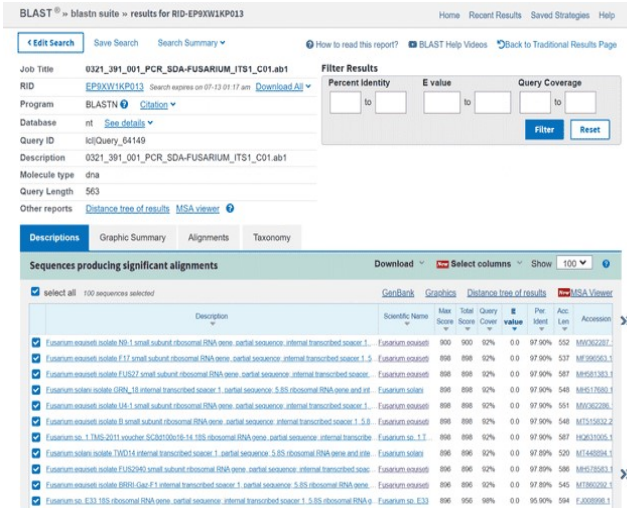

Figure 4: BLAST Analysis of 28S rRNA sequence (showing >90% homology with *Fusarium equiseti*)

Dye decolorization studies

Different dye concentrations of 0.01%, 0.02%, and 0.03% along with a control (without inoculum) were used for the dye decolorization study. The spectrophotometric analysis was performed after the 2nd, 4th, and 6th day of incubation (25 °C). The results showed a reduction in the color of the dye indicating that decolorization of the dye (0.02%) took place at the maximum of 75.23% within 6 days at 25°C (**Table 1**). Several data are available for maximum decolorization of textile effluent by two fungal isolates at 7 days of incubation period and decolorization (%) decreased gradually after 9 days **[27]**. Performance of fungus seems to be better 17, which causes 53% decolorization in 18 days with Poly R-478 dye (initial concentration., 100 mg/l).

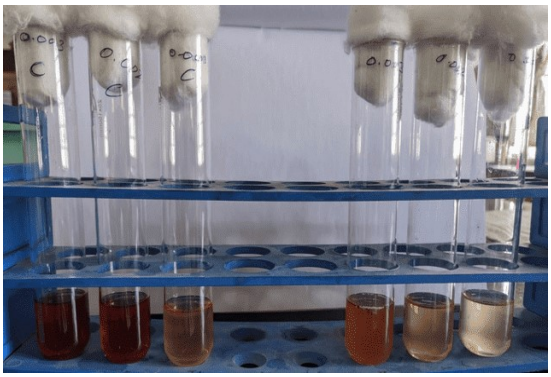

Figure 4: Dye degradation experimental setup

FTIR analysis

The degradation of dye at different concentrations was carried out in the presence of fungal isolate *F. equiseti*. The absorption peaks at 500 - 550 nm for dye decreased gradually with the increase of the exposure time and that indicates the degradation reaction. The completion of the degradation of the dyes is known from the gradual decrease of the absorbance value of the dye approaching the baseline. From the spectrum, it is evident that the fungal isolate degrades the dye at 0.02% with an absorption range of 0.141 at 800 nm at 25 °C after 72 hours (**Figure 6**). According to UV-Visible spectra, the sample degraded maximally (51.02%) at 200 nm for methyl orange. For Eriochrome Black T, the sample degraded maximally (35.12%) at 700 nm and 33.72% at 200 nm **[28]**.

The FTIR analysis was performed to get information on the transformation and molecular structure of the dye. For this 0.02% concentration of the dye samples from day 4

and day 8 along with the control were used to perform the FTIR Analysis. FTIR measurements were carried out to identify the potential changes in the functional groups caused by *Fusarium equiseti* which is responsible for the reduction of the dye (**Figures 7a, 7b & 7c**). FTIR spectrum shows a strong absorption peak at 3281cm^{-1} which indicates the presence of alcohol. The broad absorption band was observed between 1636 to 1245 cm^{-1} due to the O–H bending and C-O stretching carboxylic acid and aryl ether to aromatic ester groups A weak band was observed at 3281 cm^{-1} corresponding to O–H stretching alcohol. New medium bands were formed during incubation at 2927 cm^{-1} and 2934 cm^{-1} owing to the conversion of alcohol to alkane in the presence of *Fusarium* (**Table 2**). Similar results were obtained in the biodegradation of dye post-incubation with sprouts. The degradation products showed a peak at 1458 cm^{-1} for CH stretching (for alkynes). The biodegradation of dye was shown by the absence of the disulfide group and azo group **[29]**.

Pectinase activity

The enzyme assay to measure the Pectinase activity was performed using the test sample and control along with the citrate buffer as the substrate. The test sample contained 2ml of the fungal broth along with 10ml of the citrate buffer, this was added into Oswald's Viscometer. As reported by Semenova *et al*., (2003) **[30]** one unit of the pectinase activity corresponds to the activity resulting in a 50% decrease in the relative viscosity of 0.5%.

Table 1: Percentage of dye degradation

Sl. No.	The concentration of dye (%)	Days	Control (OD in nm)	Test (OD in nm)	D (%)
1	0.01	2	0.31	0.28	12.90
		4	0.31	0.28	
		6	0.30	0.27	
2	0.02	2	1.05	0.30	75.23
		4	0.99	0.28	
		6	0.94	0.26	
3	0.03	2	1.15	0.96	28.69
		4	1.05	0.88	
		6	0.98	0.82	

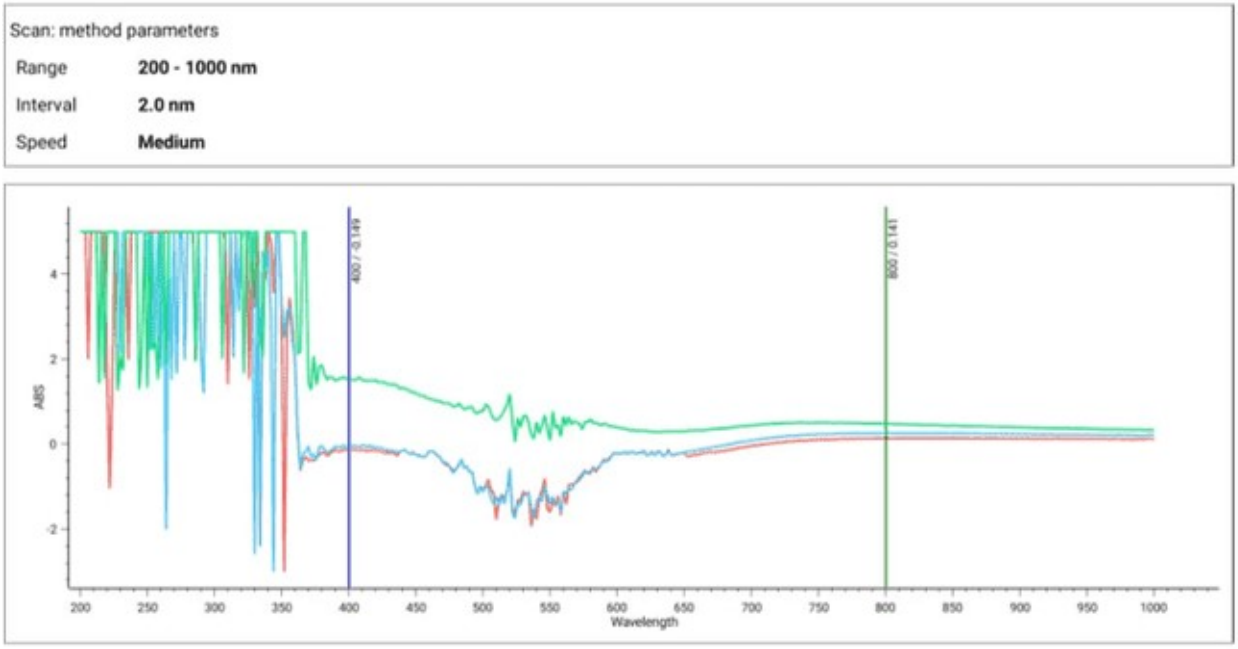

Figure 6: UV-Visible spectrum of dye treated with *Fusarium equiseti* after 72 hours. (Red - 0.02% of dye concentration; Blue - 0.03% of dye concentration; Green - 0.04% of dye concentration)

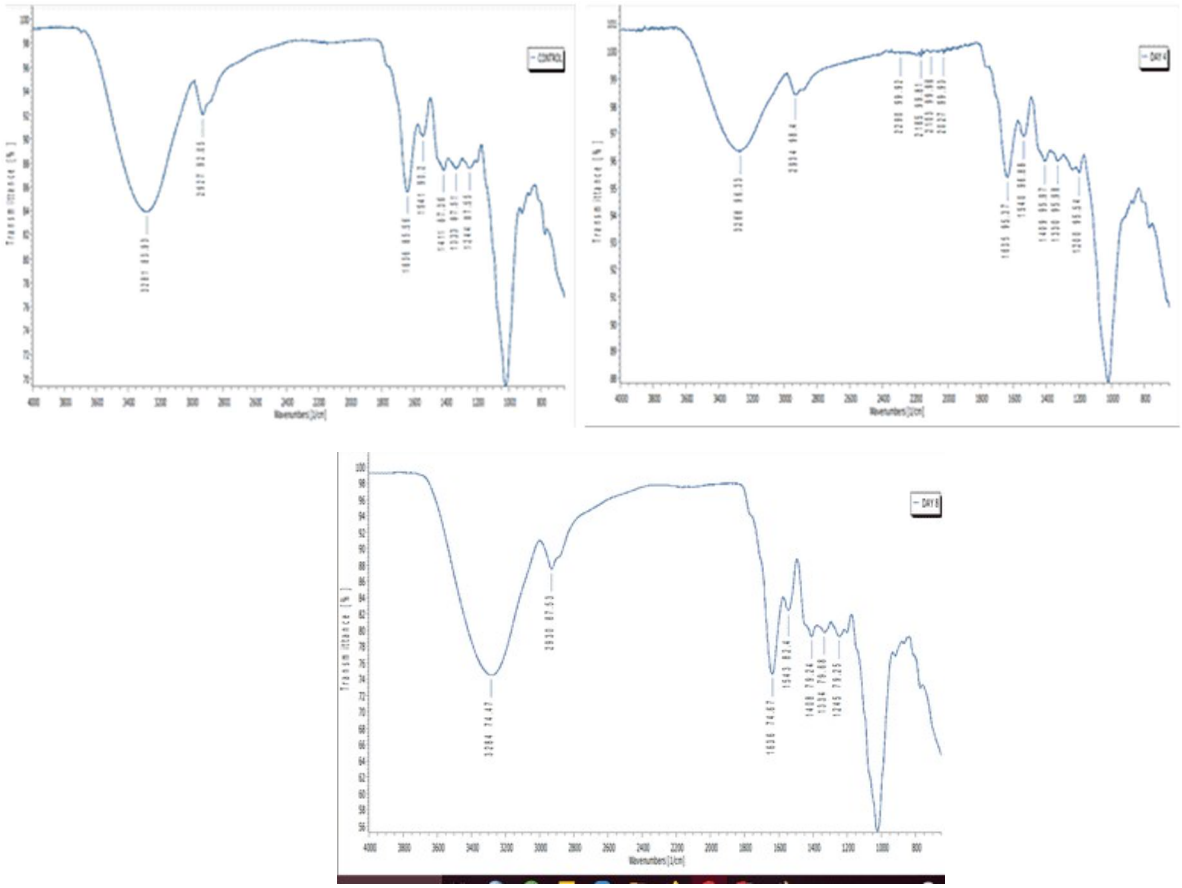

Figure: 7: FTIR spectrum of Dye degradation; 7a - Control; 7b - 4th day and 7c - 8th day Of incubation

Citrus pectin solution for 5 min at 40°C and the appropriate pH. Reduction in viscosity after 2 hours indicates an increase in pectinase activity. It was estimated to be (2.27 to 1.19) approximately 1 Unit of enzyme activity increased after 2 hours of incubation with the dye and fungal culture. Similar results with *Aspergillus niger* endopectinase were found to reduce 32·7% viscosity after 30 min and 50·0% after 60 min in the studies **[31]**.

The pH study shows that the maximum decolorization is shown when the pH is 4. This can be explained by the fact that at high pH, fungal cells transfer their protons, which leads to a decrease in the pH of the medium. Acemioglu *et al.,* 2010 proved that the increase of the removal percentage of MB with increasing pH was studied **[32]** but, the changes of adsorption and decolorization of Procion Red and Cationic Blue decreased with increasing pH **[33]**.

Table 2: FTIR Analysis of dye degradation

Sl. No.	Absorption (cm^{-1})	Appearance	Group	Frequency shift cm^{-1}	Control system	With Inoculum after 8 days
1	3550-3200	strong, broad	O-H stretching	3281-3284	alcohol	alcohol
2	1690-1640	medium	C=N stretching	1636	imine / oxime	imine / oxime
3	1550-1500	strong	N-O stretching	1541-1543	Nitro compound	Nitro compound
4	1440-1395	medium	O-H bending	1411-1408	alcohol	carboxylic acid
5	1420-1330	medium	O-H bending	1333-1334	sulfonate	alcohol
6	1310-1250	strong	C-O stretching	1244-1245	Aryl ether	Aromatic ester
7	3000-2840	medium	C-H stretching	2927 2930	alcohol	alkane

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