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RESPONSES OF FIVE SOIL BORNE FUNGI TO TOXICITY OF THREE LOCALLY SYNTHESIZED DYES

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ABSTRACT

The toxicity of three new and locally synthesized dyes [namely 3,5- dimethoxypyrimidine azo-6-methyl uracil (Dye 1), 4-ethyl 5,2,3-thiazole azo-6-methyl uracil (Dye 2), and 5-ethoxybenzothiazole azobenzoloxyphenol (Dye 3)] to five soilborne fungi: *Aspergillus niger, Fusarium* sp., *Trichoderma viride, Alternaria alternate and Penicillium chrysogenum* was investigated. It was observed that the dyes at concentrations of 0.1, 1.0, 10 and 100 (mg/L) inhibited the extension of the mycelia of each fungus to varying extent. Increases in dye concentration resulted in reduced mycelia extension. Although, there were no significant differences at $p \leq 0.05$ on the effects of each dye on the five test fungi mycelia extensions, significant differences (at $p \leq 0.05$) were observed in the effect of various concentrations of each dye on mycelia length. Additionally, *Aspergillus niger* and *Fusarium* sp. displayed capabilities of decolourizing Dye 3 which was selected for decolourization test. At the end of a four day exposure period, percentage decolourization of dye at concentrations of 0.1,1.0, 10 and 100 mg/L were 60, 60.97, 19.96 and 10.26 (%) respectively when *Aspergillus niger* was utilized while significantly lower (at $p \leq 0.05$) values, (33.33, 50.24, 10.07 and 5.25(%) were obtained on the utilization of *Fusarium* sp. for decolourization purpose. The demonstration of dye decolourization abilities and adaptation of various mycelia growth to toxic pressure of dyes, indicate possible roles these fungi could play in ameliorating the effects of these dyes in cases of accidental or indiscriminate discharges into the environment.

Keywords: Fungi, Dye, Toxicity, Decolourization

INTRODUCTION

Dyes are colouring agents used to impact colours to various substances. They are salts that carry a coloured component called the chromophore. Dyes are utilized especially in the textile industry and in other facets of life including Microbiology, Zoological as well as histological research/ diagnostic laboratories where they are employed as stains for the differentiation of cells. Also, dyes have applications in other numerous industries such as the cosmetics, pharmaceutical, food, leather, paint and printing industries (Hassan and El Nemr, 2017). A global discharge of about 280,000 metric tons has been reported as the volume of dyes released annually into the environment in the cause of usage including, synthesis, processing and applications of dyes (Almeida and Corso, 2014; Omar, 2016).

In third world countries, waste containing dyes frequently leak either deliberately or accidentally into surrounding environments mostly the terrestrial ecosystem. These leakages and subsequent persistence of waste dye (s) in environments are usually as a result of one or more of the following: inadequacies in waste treatment/disposal infrastructural facilities, poor environmental sanitation, weakness in enforcement of legislature as well as lack in orientation in regards to attitudes of eco-friendliness and sustainability. Dyes discharged into environments could possibly disrupt the balance of such ecosystems. The occurrence and persistence of dyes in the environments could pose serious threats and concern to both flora and fauna (Joshi *et al*., 2015). Hitherto, the toxicity, mutagenicity as well as the carcinogenic potentials of dyes especially those containing aromatic compounds have been, reported (Berbsten-Torralba *et al.,* 2009; Omar, 2016; Das and Das, 2017).

Fungi play a major role in the maintenance and modulation of terrestrial ecosystems. They are involved in the degradation of organic matter, biosorption of toxic elements, bio-transformations, bio- protection from pests and diseases (bio-control agents), nutrient mobilization, soil structure formation and hence overall regulation of the dynamics of physiological processes in soil environments (Bridge and Spooner, 2001; Frac *et al*., 2018). Only little information exists on non-basidiomycetes fungi that degrade dyes. Nevertheless, fungi are very efficient in metabolizing a vast array of compounds by either demethylation or through their highly oxidative and non-specific ligninolytic enzymes (Rani *et al*., 2014). This therefore formed the basis of this study to investigate the capacity of some soil borne fungi to withstand the toxicity of three dyes synthesized locally and to further determine the dye decolourization abilities of the isolates thereby ascertaining the bioremediation or biotransformation potentials of indigenous soil borne fungi.

MATERIALS AN D METHODS

Source of Dyes

Three dyes; 3,5- dimethoxypyrimidine azo-6-methyl uracil, 4-ethyl 5,2,3-thiazole azo-6-methyl uracil, and 5-ethoxybenzothiazole azobenzoloxyphenol hereafter referred to as Dye 1, Dye 2 and Dye 3 respectively were utilized in this study. The dyes were synthesized in the chemistry laboratory of the Delta State University, Abraka by Dr J. Otutu and co-

workers. The dyes were sparingly soluble in water and were all slightly brown in colour.

Source of Isolates

Five important fungal isolates including *Aspergillus niger* (organic matter decomposer) *Trichoderma yiride* (antagonist of other soil organisms especially plant pathogens), *Fusarium* sp (pathogen), *Alternaria alternata* (pathogen) and *Penicillium chrysogenum* (important in antibiotics production) were obtained from soil got from various virgin land, within the Abraka campus of the Delta State University. Top soils (5 – 10 cm depth) were collected using sterile auger, into new black polyethylene bags and transported to the laboratory immediately. One gram of each soil sample was then weighed, diluted using the ten-fold serial dilution method, after which, 0.1ml of the 10^{-3} dilution factor was inoculated aseptically into potato dextrose agar using the pour plate method. Incubation was done at ambient temperature for 2-7 days. Discrete colonies that developed were isolated and sub-cultured into slants for identification and future reference. Identification was based on cultural and microscopic morphologies with particular cognizance (reference) to Barnett and Hunter (1972). Stock cultures were then stored at 4^{o}C in a refrigerator,

Preparation of Dye Concentration (Toxicant)

Each dye (0.1g) was dissolved in 5ml of the solvent dimethyl sulfoxide (DMSO) and then the dye solution was further introduced into 95ml sterile deionized water to make a stock solution containing dye at a concentration of 1000mg/L. Subsequent ten-fold serial dilution was made up till 10^{-4} to give logarithmic concentrations of 100, 10, 1 and 0.1 (mg/L). These served as the toxicant concentrations used in this study.

Determination of the Effect of Toxicant on Mycelia Extension

Potato dextrose agar used in this experiment was prepared using 100 ml of each toxicant concentration obtained as described in the preceding section as the culture medium preparation water. Having poured the plates and allowed to set, each fungus (3mm plug) was sub-cultured onto the surface of PDA plates into which the dye concentration had been incorporated. Incubation followed at ambient temperature for 7 days. The radial mycelia extension was measured on Day 7 of incubation. Negative control plates contained appropriate concentration of DMSO (the solvent for dye dissolution) only + the organism while positive control plates contained only the organism (i.e. both dye and DMSO were excluded from medium). Percent mycelia extension in plates containing various dye concentration was calculated thus:

$$\% \text{ mycellial extension} = \frac{ME_f - (ME_{dm} - ME_{dmd})}{ME_f} \times 100$$

Where,

ME_{dm} = Mycellia extension in medium containing DMSO and organism.

ME_{dmd} = Mycellia extension in medium containing DMSO, dye and organism

ME_f = Mycellia extension in medium containing only organism

Whereas, $ME_f - (ME_{dm} - ME_{dmd})$ represented the actual mycelial extensions in media containing dye

Dye Decolourization Experiment

This experiment was done, to determine the capability of two fungal isolates (*Aspergillus niger* and *Fusarium*) in decolourizing the most potent dye. The two fungi were selected based on performance in mycelia extension in dye presence as observed in the preceding experiment. The standardized inoculum (5.0 mm fungal plug) was obtained and exposed to the varied dye concentration at pH 5.5. Each set-up was allowed to stand at ambient temperature under shaken conditions (150 rpm) and at regular intervals of Day 0, 1, 2, 3 and 4 optical densities were measured accordingly. The percentage decolourization was calculated as shown below. Also, a mathematical projection (i.e. modelling) of the time for complete dye decolourization (100% decolourization) by each isolate under the tested conditions was made using Ms-Excel application

Percent decolourization = $(OD_i - OD_f / OD_i)100$

Where,

OD_i = Initial optical density

OD_f = Final optical density

While the regression equation ($y = mx + C$) was used to predict the time for complete decolourization

Where,

y = 100% decolourization

x = predicted time for the complete decolourization

m = gradient of slope

C = intercept

Statistical Analysis

Data obtained were from duplicate samples and were analyzed using statistical tools including ANOVA, t-test, determination of mean, correlation and regression

RESULTS

The results of effects of each dye on the radial mycelia extension of various isolates are represented in figures 1-3. Results obtained revealed that the radial mycelia extension of each isolate decreased as the dye concentrations increased. At 0.1mg/L of Dye 1, radial mycelia extensions were 11.5, 13.2, 22.9, 15.8 and 15.5 (mm) for *Trichoderma viride*, *Alternaria alternate*, *Penicillium chrysogenum*, *Aspergillus niger* and *Fusarium* sp. respectively. On exposure to the highest tested concentration (100mg/L), lower mycelia lengths of 5.5 mm (*Trichoderma viride*), 3.17 mm (*Alternaria alternate*), 2.55 mm (*Penicillium*), 14.5 mm (*Aspergillus niger*) and 12.5mm (*Fusarium* sp.) were observed. Again, cultivation of each fungus in concentrations of Dye 2 that ranged from 0.1 to 100 (mg/L) resulted in radial mycelia extensions that accordingly, reduced from 7.5 to 5.0 (mm), 7.8 to 3.4 (mm), 16.5 to 2.5 (mm), 18.5 to 16.5 (mm) and 18.5 to 12.5 (mm) in *Trichoderma viride*, *Alternaria alternate*, *Penicillium chrysogenum*, *Aspergillus niger* and *Fusarium* sp. respectively. Similarly, radial mycelia lengths in *Trichoderma viride*, *Alternaria alternate*, *Penicillium chrysogenum*, *Aspergillus niger* and *Fusarium* sp. cultivated in medium containing 0.1 mg/L of Dye 3 were 6.5,8.3, 12.5, 16.5 and 16.5 (mm) respectively while at 100mg/L, 5.5, 3.6,

4.0, 13.5 and 10.5 (mm) were the radial mycelia lengths obtained. Although, there were no significant differences at $p \leq 0.05$ on the effects of the three dyes on the five test fungi radial mycelia extensions, significant differences (at $p \leq 0.05$) were observed in the effect of various concentrations of each dye on mycelia length.

Although, no dye concentration tested was able to completely inhibit the extension of the mycelia of the fungi isolates, lower mycelia lengths were observed in plates that contained dye in comparison to positive control plates. These differences were significant at $p \leq 0.05$ using ANOVA.

Furthermore, radial mycelia extensions were negatively correlated to dye concentrations indicating an inverse relationship between the variables. In cases of Dye 1, 2 and 3 the respective correlation values were -0.5332, -0.40919 and -0.4072 (*Trichoderma viride*), -0.6035, -0.31285 and -0.4432 (*Alternaria alternate*). -0.6003, -0.4872 and -0.3782 (*Penicillium chrysogenum*), -0.6530, -0.5261 and - 0.7569 (*Aspergillus niger*) as well as -0.8324, -0.7296 and -0.6579 (*Fusarium*s sp.). Similar responses were displayed by all the fungi isolates on exposure to each dye with exception of *Aspergillus niger*; as the spread of its mycelia were rather stimulated by the various dyes at concentrations of 0.1 and 1.0 mg/L. Indeed, they were a little longer than the mycelia length of positive control isolate. However, at every given dye concentration, there was no significant difference ($p \leq 0.05$) in the corresponding resultant mycelia lengths of the five fungi.

Fungi responses to the three dyes in terms of inhibition of isolates' mycelia extension were ranked thus: *Aspergillus niger* ≤ *Fusarium* sp. ≤ *Trichoderma viride* ≤ *Alternaria alternata* ≤ *Penicillium chrysogenum* (i.e. *Aspergillus niger* was least affected as depicted in Table 1 by which the results of the percentage mycelia extentions of test isolates in the presence of various dye concentrations are illustrated. The results obtained (Table 1) indicated that, percent mycelia extensions dropped progressively as dye concentration increased from 0.1 to 100 (mg/L). The toxic pressure exerted by the dyes on mycelia of various test fungi was in the order Dye 3 > Dye 2 ≥ Dye 1.

Decolourization experiment revealed that *Aspergillus niger* and *Fusarium* sp. had the ability of decolourizing Dye 3 to varying degree as represented in Figures 4 and 5. Although, (dye decolourization increased with increases in exposure period, it was observed that decolourization only increased with increasing concentration to an extent (1mg/L). At initial dye concentration of 10mg/L and beyond decolourization rate reduced.

The projections made concerning the time required for complete decolourization of dye, as shown in Table 2, indicated that whereas, both isolates would be able to completely decolourize the dye at all given test concentrations, it will take *Fusarium* sp. a significantly longer period (at $p \leq 0.05$ using t-test) than *Aspergillus niger*. While, it will likely take *Aspergillus niger* a period ranging from 6 to 35 days for the complete decolourization of Dye 3 at concentrations ranging from 0.1 to 100 (mg/L), the time frame required by *Fusarium* sp. for same purpose would range from 7 to 63 (days). An initial reduction in required time was observed as the dye concentration increased to an extent (from 0.1 to 1 (mg/L)). Thereafter, required time increased with increasing dye concentration.

DISCUSSION

The significantly shorter mycelia lengths observed in fungi exposed to the varied dye concentrations when compared to controls, is an indication of the probable toxic pressure effects of the dyes to the test isolates. The radial mycelia growth inhibitions are likely due to the complex aromatic structures of the dye. Also, the fact that radial mycelia extensions responses were influenced by varying dye concentrations is implicative of the negative or deleterious impacts of the test dyes especially at high concentrations. Similar observations have been made by Almeida and Corso (2014); Das and Das (2017). Importantly, this similar behaviour of all test fungi including the pathogens, parasite antagonist and organic matter degrader suggests that all of these fungi possessed similar response strategy to the dyes which could either be by biosorption, biodegradation or both (Przystas *et al*., 2015; Omar, 2016). Perhaps, the similarity observed in the manner of response of the five fungi to the toxicity of the dyes may be due to the relative similarities in the molecular structures of the dyes. Also, the pattern of response demonstrated by the isolates could probably be attributed to the possession and expression of same gene (s) in all isolates enabling the transformation of the dye structures to lesser toxic forms.

The inhibitory action of the dyes noticed in this study may be as a result of their complex chemical composition including the presence of aromatic / phenolic components. Implicatively, the higher dye concentrations had greater effects in the role of inhibition of the fungi metabolic activities.

Again, the non-absolute inhibitions of radial mycelia extensions observed at all tested concentrations, may be due to gradual adaptation to the xenobiotics. These adaptations may include the acquisition of ability to accumulate the compound in fruiting bodies, innate dye resistance ability or probably due to ability to secrete enzymes with tendencies of breaking down the dye components. It could also be attributed to resistances of test fungi to by- products of transformations of these dyes in cases where such transformations might have taken place. Similar reports have been made by Hartikainen *et al*., 2016 and Hadibarata *et al*., 2013.

The observed pattern in dye decolourization suggest strongly that the mechanism leading to dye decolourisation by these ascomycetes more or less involved bioconcentration of dye into the cytoplasm or biosorption into fruiting bodies. Although, biotransformtion processes such as mineralization of the dye may not be ruled out (Berbsten-Torralba *et al*., 2009). The decolourization capability demonstrated by the strains of *Aspergillus niger* and *Fusarium* sp. utilized in this study, further corroborates various works on the use of micro-fungi including the brown rot fungi in the decolourization of different kinds of dyes (Berbsten-Torralba, 2009; Almeida and Corso, 2014; Omar, 2016; Hartikainen *et al*., 2016; Das and Das, 2017). Previously, emphasis had been on white rot

fungi such as *Phanerochaete chrysosporium and Trametes versicolor* which produce ligninolytic enzyme enabling the breakdown of a vast array of complex xenobiotics like synthetic azo dyes (Das and Das, 2017). Hence, the decolourization efforts displayed by the two fungi (*Aspergillus niger* and *Fusarium* sp.) increases the prospects in areas of the development of bioagents for the treatment of dye waste. The results obtained suggest strongly that the major mechanism of decolourization was likely by biosorption into fungal biomass. Saturation of the sites of accumulation in the biomass might have been responsible for the reductions noticed in the rate of decolourization

The adaptive responses displayed by these soil borne fungi utilized in this study, demonstrate that these organisms could be employed in the remediation of sites contaminated by these dyes or at least there is a pointer that the isolates could be useful in ameliorating the effects of these dyes in cases of accidental or indiscriminate discharge into the environment. Further studies are however necessary to optimize these fungi abilities and hence establishing the feasibility of these assertions in an industrial setting.

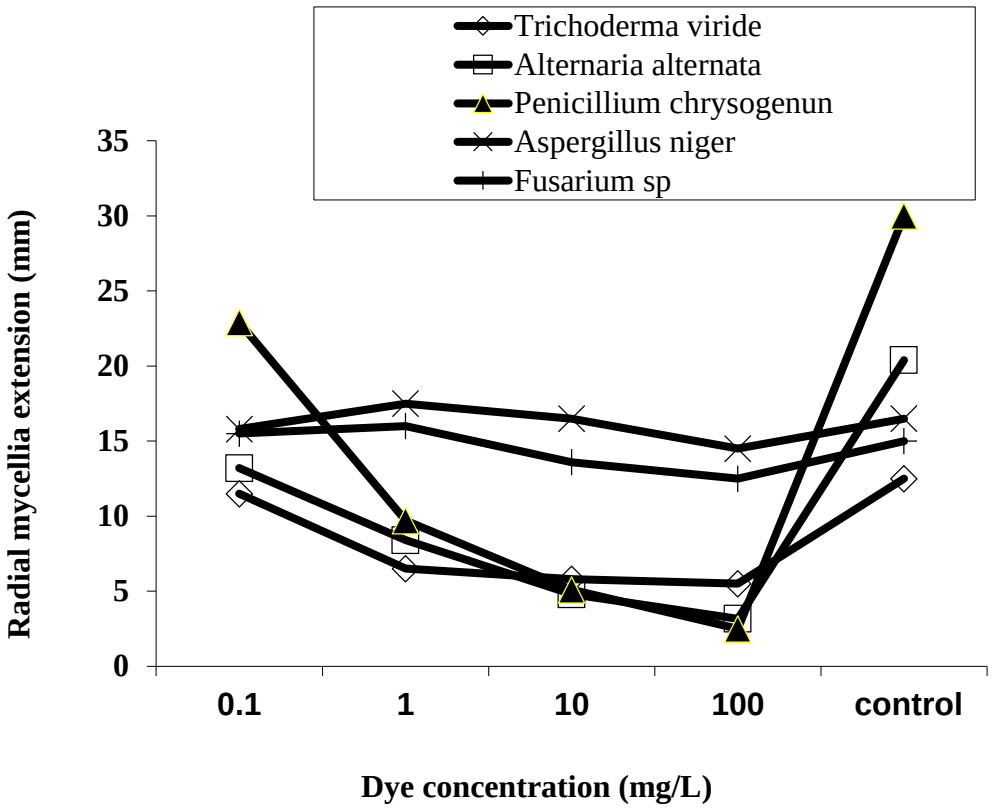

Fig. 1: Effect of Dye 1 on mycellia extension of various isolates

Key: Dye 1 = 3,5- dimethoxypyrimidine azo-6-methyl uracil

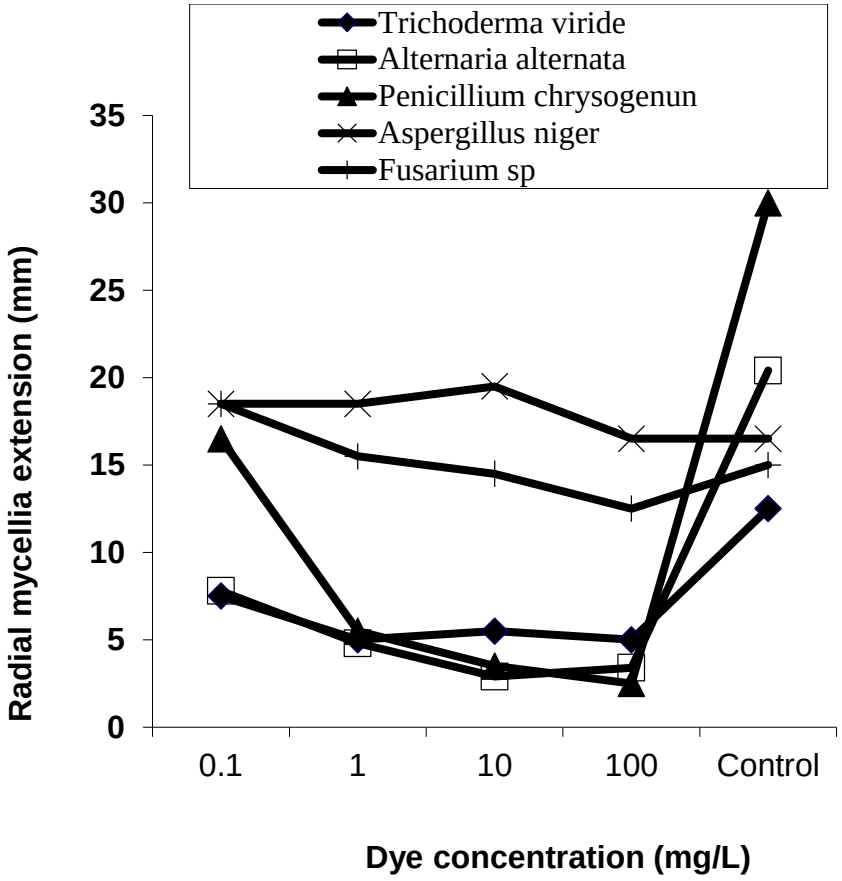

Fig. 2: Effect of Dye 2 on mycellia extension of various isolates

Key: Dye 2 = 4-ethyl, 5,2,3-thiazole azo-6-methyl uracil

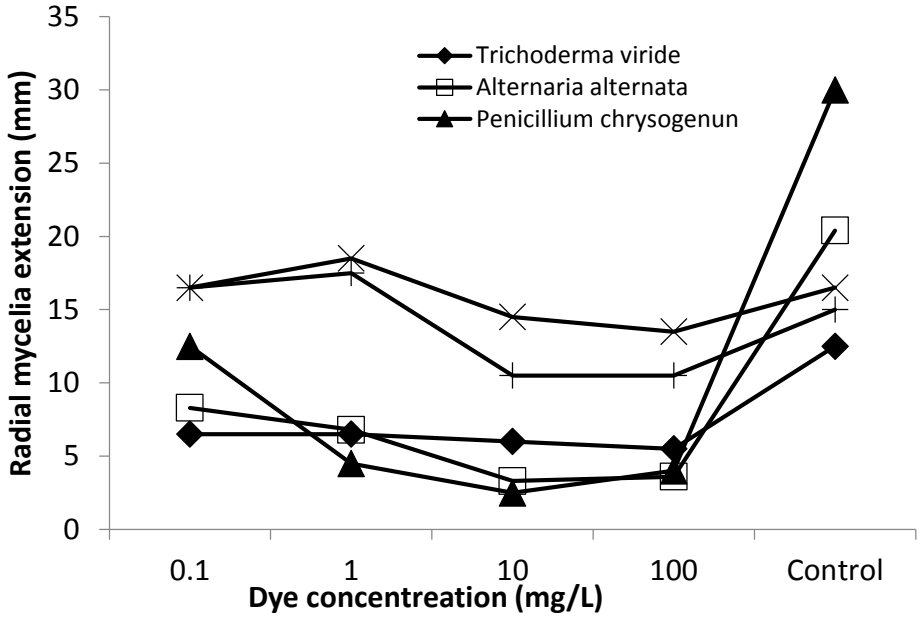

Fig.3: Effect of Dye 3 on mycelia extension of various isolates

key: Dye 3 5-ethoxybenzothiazole azobenzoloxyphenol

Table 1: Percent mycelia extension of isolates exposed to various dyes

Fungal Isolate	Dye conc.	Percent mycelial extension		
		Dye 1	Dye 2	Dye 3
Trichoderma viride	0.1	92	60	52
	1.0	52	44	52
	10	46.4	40	48
	100	44	44	44
Alternaria alternate	0.1	64	38.24	40.67
	1.0	41.18	23.53	38.64
	10	23.53	14.22	16.18
	100	15.54	2.94	13.73
Penicillium chrysogenum.	0.1	76.33	55	17.65
	1.0	32.33	18.33	11.67
	10	17	11.67	8.33
	100	0.33	8.33	13.33
Aspergillus niger	0.1	104.24	112.12	100
	1.0	106.06	112.12	112.12
	10	75.76	118.18	87.87
	100	87.88	100	81.82
Fusarium sp.	0.1	96.67	123.33	110
	1.0	93.33	103.33	116.67
	10	96	96.67	70
	100	83.33	83.33	70

Dye 3 = **5-ethoxybenzothiazole azobenzoloxyphenol**

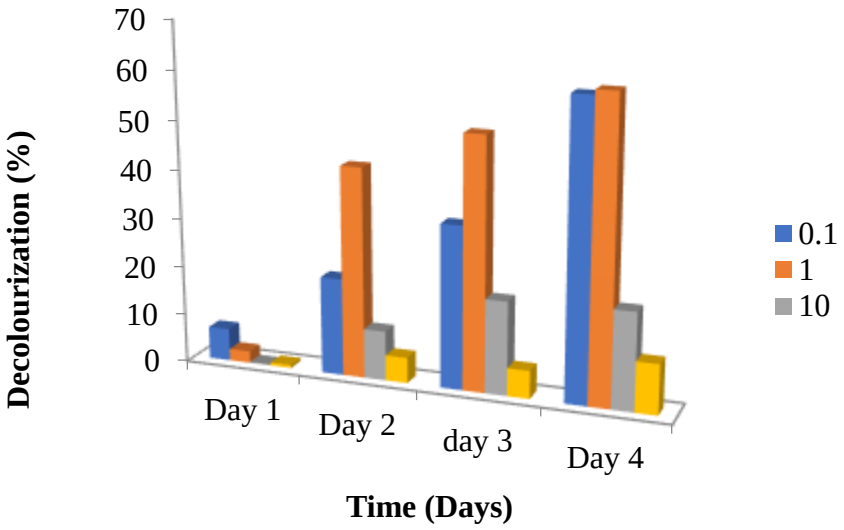

Figure 4: Decolourization of dye 3 by *Aspergillus niger*
Key: Dye 3 =5-ethoxybenzothiazole azobenzoloxyphenol

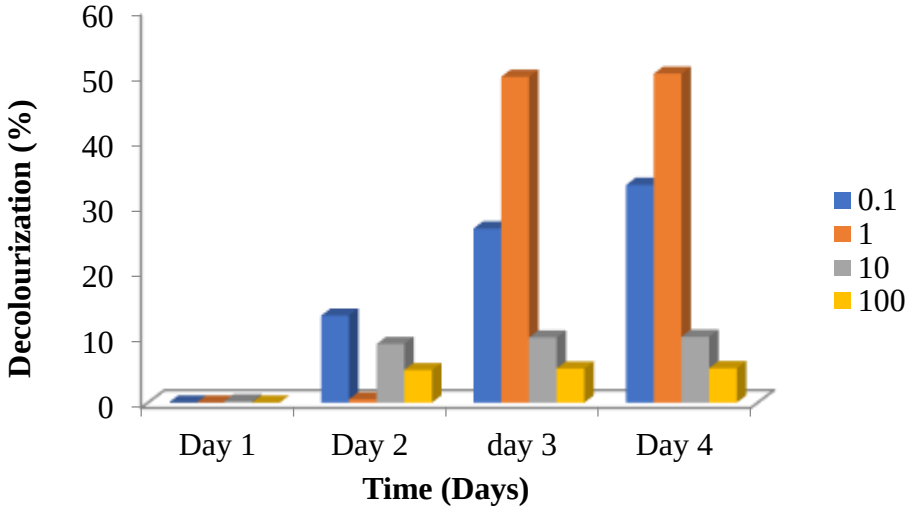

Figure 5: Decolourization of dye 3 by *Fusarium* sp

Key: Dye 3 =5-ethoxybenzothiazole azo-6-benzoloxyphenol

Table 2: Time projections made for the complete decolourization of Dye 3.

Dye concentration (mg/L)	Time projection (Days)	
	Aspergillus niger	*Fusarium* sp.
0.1	7	10
1.0	6	7
10	16	33
100	35	63

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