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Capacity of Growing, Live and Dead Fungal Biomass for Safranin Dye Decolourization and Their Impact on Fungal Metabolites

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ABSTRACT

Background: In recent years, bio-treatment took attraction in removing the unwanted color and toxicity of dyes than other conventional treatment processes. **Objective:** our investigation has the purpose to verify the decolorization potential of growing, live and dead biomass of *Aspergillus flavus* and *Mucor hiemalis* upon the safranin dye, and to conclusively prove the role of ligninolytic enzymes secreted by the selected fungal strains to improve the rate of decolorization. **Results:** Decolourisation % increased, 69.71, 85.93 and 86.70% for growing biomass of *Aspergillus flavus*, 39.81, 57.59 and 68.73% for growing biomass of *M. hiemalis* with increasing safranin concentration at 125, 250, 500 µg respectively. Safranin bioremoval rate by growing fungal biomass was higher (69.71 and 36.50%) than dye biosorption by living (45.10 and 36.50%) and dead (20.50 and 28.56 %) biomass of *A. flavus* and *M. hiemalis* respectively in the same incubation time and at 125 µg/100mL concentration and at other concentration. The biosorption capacity increased sharply with increasing incubation time until 10 days, and then negligible biosorption was observed. The biosorption capacity of *A. flavus* was more effective than of *M. hiemalis*, where decolorization % with using *A. flavus* at 12 days was 69.72, 85.98 and 86.71% compared with *M. hiemalis*, where decolorization % was 40.11, 58.24 and 68.77 % at 125, 250 and 500 µg/100mL respectively. The enzymes, lignin peroxidase and laccase were estimated during the decolorization of safranin dye by *A. flavus* and *M. hiemalis*. In *A. flavus* maximum decolorization % was 89.35 at safranin concentration 500 µg/100mL with the addition of lignin compared with the decolorization% 85.93 without lignin addition. Presence of safranin dye in fungal growth medium suppressed the synthesis of certain fatty acids such as caprylic, linoleic, arachidic and behenic specially at high concentration of safranin (500µg/100mL). **Conclusion:** *A. flavus* and *M. hiemalis* will contribute to the decolorization and degradation of effluents containing safranin dye and their action will reduce the pollutant discharge and toxicity related to the presence of dyes.

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INTRODUCTION

Globally, it is estimated that over 7×10^5 ton and approximately 10,000 different dyes and pigments are produced annually world-wide, it is estimated that 2-50% of them are lost into wastewaters, causing environmental contaminations (Deveci *et al*., 2004). Discharge of dyes into the aquatic or other environment causes serious problems, since they may affect the photosynthetic activity of hydrophytes by reducing light penetration intensity (Aksu *et al*., 2007) and also they may be toxic to aquatic fauna and flora due to their breakdown products such as metals and chlorides (Hao *et al*., 2000; Dhaneshvar *et al*., 2007). The presence of safranin causes several acute effects on health (like irritation to mouth, throat and stomach with effects including mucous build up, irritation to the tongue and lips and pains in the stomach, which may lead to nausea, vomiting and diarrhea, irritation to the eyes, with effects including: tearing, pain, stinging and blurred vision, redness and itchiness of skin) (Rejniak and Piotrowska, 1966). Safranin has comparable mutagenic effects on *Drosophila melanogaster* (Rey, 2012). Due to its structural stability,safranin is difficult to biodegrade (Kumar., 2007; Chowdhury *et al*., 2010).

Contamination by dyes represent a relevant issue associated with several industries. Dyes, even at very low concentrations, reduce oxygen solubility and are often toxic and recalcitrant; moreover, these chemicals are toxic, carcinogenic or mutagenic for various organisms (Nirmalarani and Janardhanan, 1988; Mathur and Bhatnagar, 2007). Hence, removal of these dyes from the effluents is necessary. Various techniques have been

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employed for the treatment of dye bearing industrial effluents, which usually come under two broad divisions including a biotic and biotic methods. To remove dyes, the physical and chemical methods like adsorption, photolysis, flocculation, chemical precipitation, chemical oxidation and reduction, electro-chemical treatment and ion-pair extraction were extensively used (Zhang *et al.*, 2002). Of the different biological methods, bioaccumulation and biosorption have been demonstrated to possess good potential to replace conventional methods for the removal of dyes (Malik, 2004).

Recently a number of studies have focused on some microorganisms capable of biodegrading and absorbing dyes from wastewater. A wide variety of microorganisms are reported to be capable of decolonization of dyes (Chang and Kuo, 2000; Ebency *et al*., 2013; Kumar *et al*., 2013; Natarajan *et al*., 2013; Ramamurthy and Umamaheswari , 2013; Sewgil and Sawan,2013). However, bacterial degradation have some limitations and in recent years, there has been an alternative research on fungal decolourization of dyes present in wastewaters, and it is turning into a promising alternative to replace or supplement for present treatment processes (Ramya *et al*., 2007). The importance of fungi and their enzymes in the dye degradation has been well appreciated globally, because of their potential use in detoxification and degradation of dyes (Assadi *et al.*, 2003). Many genera of fungi either in living or dead form have been employed for the dye decolourization (Prachi and Anushree, 2009; Mahmooda *et al*., 2014). Saranraj *et al*. (2010) reported that the fungal isolates like *Aspergillus niger, A. flavus, A. fumigatus, Fusarium oxysporum, Penicillium chrysogenum, Trichoderma viride* and *Mucor* sp. were used as a good microbial source for waste water treatment. *Mucor mucedo* decolourised 78% of the crystal violet and 65% of malachite green (Moturi and Singara-Charya, 2009). The fungus *Trichoderma harzianum* has also been reported for the degradation of dyes (Singh and Singh, 2010). Microorganisms not only able to decolourize dyes but also detoxify it (Adedayo *et al*., 2004; Kumar *et al*., 2007). Dyes are removed by fungi through biosorption, biodegradation, bioaccumulation and enzymatic mineralization (Wesenberg *et al*., 2002). Laccase, lignin peroxides, manganese peroxidase and H_2O_2 dependent peroxidases are functional extracellular enzymes by fungi in biodegradation of lignin and dyes (Arora and Gill, 2000). Production of these enzymes from white-rot fungi has been well documented. However, in recent years, there are several reports of these ligninolytic enzymes being produced from other fungi like, *Aspergillus, Fusarium and Penicillium* (Sahoo and Gupta, 2005; Pant and Adholeya, 2007; Deepak and Alok, 2009).

Adsorption of dyes to the microbial cell surface is the primary mechanism of decolourization of dyes (Knapp *et al*., 1995). The ability of white rot fungus *Phanerocheate chrysosporium* to degrade dyes has been reported with enzymes involved in lignin degradation, such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Robinson *et al*., 2001). Our investigation has the purpose to verify the decolorization potential of growing, live and dead biomass of certain fungi upon the safranin dye, and to conclusively prove the role of ligninolytic enzymes secreted by the selected fungal strains to improve the rate of decolorization.

MATERIAL AND METHODS

Dye used and Decolourization assay:

The synthetic safranin O dye, chemical formula $C_{20}H_{19}N_4Cl$, molecular weight 350.8 and molecular structure was showed in Fig. (1). Decolourization assay was measured in the terms of percentage decolourization using UV-Spectrophotometer. The concentration of dye in the supernatant was determined by reading the absorbance at 521 nm. The decolourization percentage was calculated from the following equation, Decolourization % = (Initial OD-Final OD/ Initial OD) x 100. Dye containing medium used as blank control medium contained both dye and medium without fungal inoculation to observe any reaction of medium with dye.

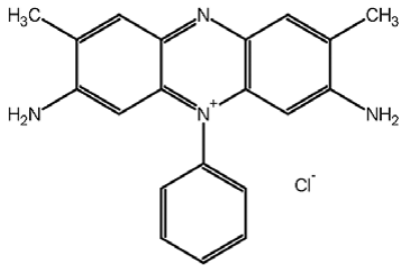

Fig. 1: Chemical structure of safranin O (3,7-Diamino-2,8-dimethyl-phenylphenazinium chloride).

Growth and Diagnostic criteria of Aspergillus flavus and Mucor hiemalis:

The fungal strains were isolated from the soil of dye contaminated industrial site located and identified as *Aspergillus flavus* and *M. hiemalis* according to Schipper (1978) Raper and Fennell (1973), Domsch *et al*. (1980). *Aspergillus flavus* and *M. hiemalis* were cultivated on Czapek Dox agar medium ($NaNO_3$, 2.0 g, K_2HPO_4, 1.0 g, KCl, 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.5 g, $FeSO_4 \cdot 7H_2O$, 0.001g, sucrose, 20 g, agar, 20 g/L, pH was

adjusted at 6.5.) supplemented with different concentrations of safranin dye (125, 250, 500 and 1000 µg/100mL) incubated for 7 days at 28°C, diagnostic criteria were achevied by using software for image analysis at the Biology department, faculty of Science, Jazan University KSA. Dry weight of *A. flavus* and *M. hiemalis* was measured after their growth in potato dextrose broth medium supplemented with different concentrations of safranin dye (125, 250 and 500 µg/100mL) for7 days at 28°C.

Biosorption with live and dead biomass:

Disc (0.6 mm) of actively margin of *A. flavus* and *M. hiemalis* colonies inoculated into 100 ml of sterile Czapek-Dox medium in 250 ml Erlenmeyer flaks and incubated at 28°C for 7 days. Mycelium developed was separated by filtration through Whatman. No.1 filter paper and washed with generous amount of de-ionized water until free from the media components. The washed mycelial pellets were subjected as a live biomass or autoclaved and dried as dead biomass to safranin biosorption. Various concentrations of working dye solutions (125, 250 and 500 µg/100mL) were prepared. To the autoclaved aqueous solutions, 400 mg of adsorbent (live or dead biomass) was added and kept in incubator at 28°C for 8 days and the decolourization of dye was assayed (Savitha and Savitha, 2005).

Biosorption with growing biomass:

Czapek-Dox medium supplemented with various concentrations of working dye solutions (125, 250 and 500 µg/100mL) was autoclaved, then inoculated with fungal mycelia/spores and incubated for 8 days or at different incubation period at 28°C in order to determine the decolorization % of safranin at different incubation period.

Lignin as inducer to safranin decolourization and ligninolytic enzymes detection:

Czapek-Dox medium supplemented with various concentrations of working dye solutions (125, 250 and 500 µg/100mL) was autoclaved. Then, 0.2 g % of lignin was added to growth medium, in order to test the ability of the selected isolates to produce ligninolytic enzymes which enhance the decolourization of used dye. Flasks containing medium were inoculated selected isolates (*A. flavus* and *M. hiemalis*) and then incubated at 28ºC for 8 days. The decolourization of dye and ligninolytic enzymes were assayed. The metabolized medium after incubation period was filtrate through filter paper containing charcoal to remove any colour of dye, and used for assay of two enzymes lignin peroxidase (LIP) and laccase (phenol oxidase). LIP activity was assayed using veratryl alcohol as a substrate. LIP catalyzes the oxidation of veratryl alcohol by H_2O_2 to veratryl aldehyde. The aldehyde was absorbed strongly at 310 nm after incubation for 1 h at 37 ºC. The reaction medium contained: 1 mL of the enzyme solution (culture filtrate), 0.2 mL of 2 mmol/L veratryl alcohol, 0.2 mL of 0.4 mmol/L H_2O_2, 0.2 mL of 0.25 mmol/L tartaric acid.

Laccase activity is determined by the oxidation of 2,2'-azino-bis (3-ethylthiazoline-6-sulfonate), i.e., ABTS at 37ºC at A420 (Buswell and Odier, 1987). One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 mMol of ABTS per minute. Reaction mixture of 1ml contained 2 mM of ABTS in a Mcllvaine buffer (pH 5.0). To the assay mixture,100 µl of centrifuged extracellular supernatants were added. The enzymatic activity was estimated in IU by monitoring the adsorbance change at 420 nm (ABTS), Є=36 $Mm^{-1}cm^{-1}$ by spectrophotometer (JENWAY, Model 6300, EU) at 30ºC.

Analysis of fungal fatty, organic and phenolic acids:

Mycelium (5 g fresh weight) of *A. flavus* and *M. hiemalis* (cultivated on Czapek-Dox broth medium containing containing two different concentrations of safranin 125 and 500µg/100mL) was grinded in 10 ml Chloroform : Methanol (2:1 v/v), then filtered and concentrated into 1 ml. The concentrated extract was placed in Gas Chromatography (GC) auto-sampler vials until they were analyzed using Shimadzu GCMS-QP 5050 A. software class 5000. Searched library: Wiley229 LIB. Column: DB1, 30m, 0.53 mm ID; 1.5 µm film. Carrier gas: Helium (flow rate 1 ml/min.). Ionization mode: El (70 ev). Temperature program: 70ºC (static for 2 min) then gradually increasing (at a rate of 2°C /min) up to 220ºC (static for 5 min). Detector temperature was set at 250ºC and injector temperature at 250ºC. The chromatographs were compared and individual peaks were identified by comparing mass spectra to the library references of fatty acids. Also, mycelium extract was put into 1 ml amber HPLC vials (Fisher) and stored at -20 ºC until processed. Organic and phenolic acids were measured using High Performance Liquid Chromatography (HPLC). An HPLC (Waters 600E System controller) with a fluorescence detector (Waters 470) and an auto-sampler (Waters 712 WISP) were used, at Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University Cairo, Egypt.

RESULTS AND DISCUSSION

Safranin Decolorization:

Physiological activity of fungi can conveniently be assayed according to decolorization of synthetic dyes. Such dyes have been widely used as model compounds to monitor the self-cleaning capacity of waters. To solve

the problem of environmental pollution, the decolorization/biosorption of dyes wastewater is a major aspect of research. The applied fungi have shown positive results for dye degradation/decolourization, as was indicated by the change and disappearance of colour of safranin dye from growth media at different their concentrations compared with the uninoculated growth medium containing 250µg safranin (Fig.2). Adsorption of the dye to the mycelium was often observed with a naked eye in the cultures, as reported in other studies, and the subsequent bleaching of the mycelium suggested a two step removal of the dye, including adsorption followed by degradation of the adsorbed dye (Wang, 1998). Adsorption of dyes to the microbial cell surface is the primary mechanism of decolourization (Knapp *et al*., 1995). In our study, the adsorption of safranin by the growing fungal mycelium was observed, as it was confirmed by the change in the colour of colony surface and reverse surface of *A. flavus* and *M. hiemalis* at different concentrations of safranin (Fig.3), the colour of colonies increased with increasing safranin concentration. Safranin O was absorbed on the surface of the biosorbent *Fomitopsis carnea* (Maurya and Mittal, 2013), *A. wentii* (Khambhaty *et al*., 2011). Muthezhilan *et al*. (2008) found that *Aspergillus ochraceus*, *A. niger*, *A. flavus*, *Rhizopus stolonifer Mucor racemosus*, shows good decolorization activity to methylene blue, gentian violet, crystal violet, cotton blue, sudan black, malachite green, methyl red and corbol fuchsion.

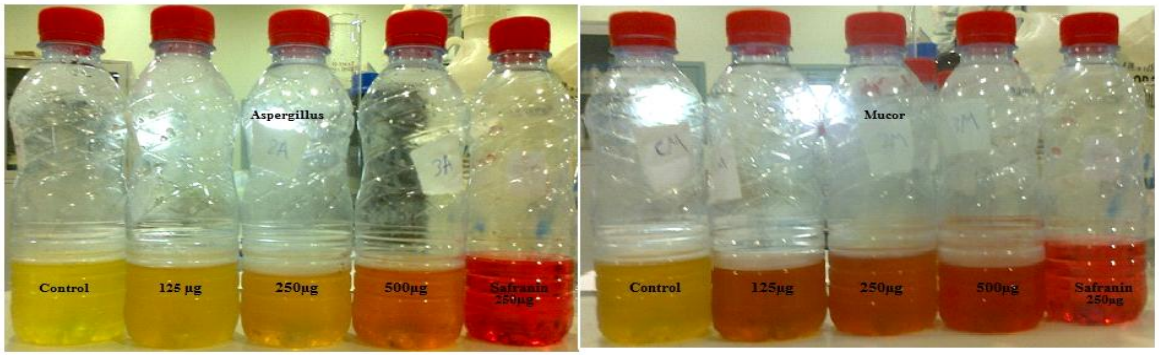

Fig. 2: Filtrate metabolized medium of growing *A. flavus* and *M. hiemalis* on growth medium supplemented with different concentrations (µg/100mL) of safranin dye. Control, Filtrate metabolized medium of growing medium without safranin, Uninoculated growth medium with Safranin 250µg used as standard control.

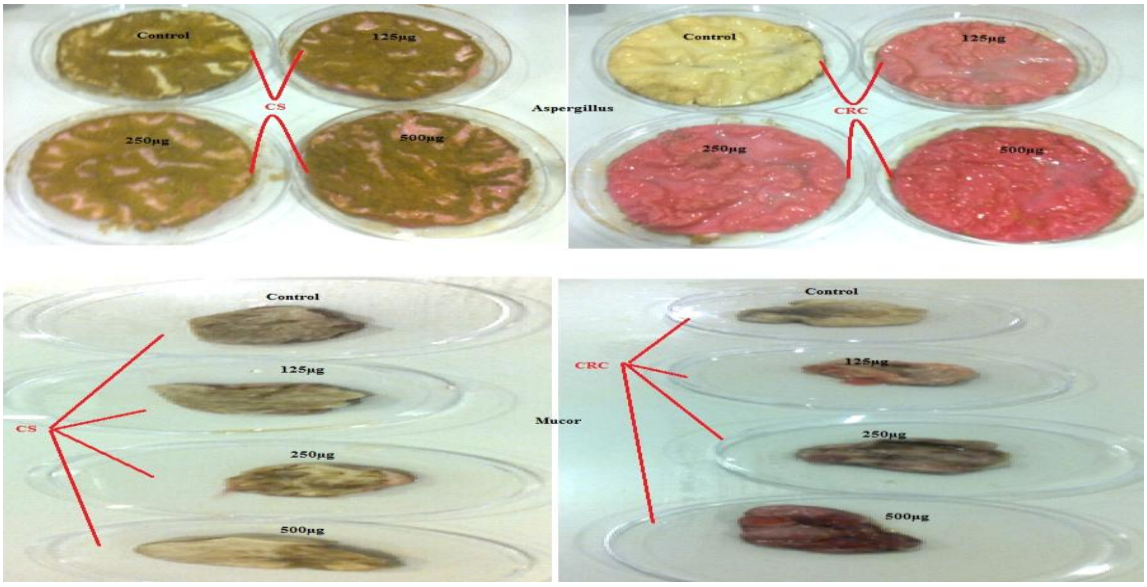

Fig. 3: Color of colony surface (CS) and colony reverse colour (CRC) of *A. flavus* and *M. hiemalis* at different concentrations of safranin growing on broth medium. Control, growth medium without safranin.

In the current study, *Aspergillus flavus* and *M. hiemalis* showed decolourisation activities of safranin indicating the role of mycelial biomass responsible for the decolourisation of the dye. Decolourisation % increased, 69.71, 85.93 and 86.70% for growing biomass of *A. flavus*, 39.81, 57.59 and 68.73% for growing biomass of *M. hiemalis* with increasing safranin concentration at 125, 250, 500 µg respectively (Table 1).

Sewgil and Sawan (2013) reported that, increasing safranin dye concentration, the removal rates decreased. In the present study dye bioremoval rate by growing fungal biomass was higher (69.71 and 36.50%) than dye biosorption by living (45.10 and 36.50%) and dead (20.50 and 28.56 %) biomass (*A. flavus* and *M. hiemalis* respectively) in the same incubation time and at 125 µg/100mL concentration and at other concentration (Table 1), it was similar to the earlier findings of Ulkuye (2013). Gopalakrishnan and Sellappa (2011) stated that decolorization rates for autoclaved biomass are not greater than those of living biomass. Also Ramalakshmi *et al*. (2011) showed that live mycelia of *Alternaria raphani* is an effective adsorbent for the removal of RB5 from aqueous solutions compared to dead biomass. Decolourisation of textile dyes with using efficacy *of A. flavus* in decolourisation of textile dyes was tested (Raju *et al.,* 2007). Recently, Mahmooda *et al.* (2014) reported that dead biomass of *Aspergillus flavus* shows maximum biosorption (53.62%) of methyl orange.

Increase in the incubation time resulted in increased growth as well as mycelial biomass and no toxic effect of safranin was noticed. At the same time the decolourisation begin with the growth of tested fungi at two days of incubation period (Tables 2&3). The biosorption capacity increased sharply with increasing incubation time until 10 days, and then negligible biosorption was observed, this may be due to fungal growth. According to Saranraj *et al*. (2010) *Penicillium chrysogenum* have the capacity to completely decolourized the dye Direct Black-E within 12 days, while *Aspergillus niger* completely decolourized the Congo Red within 6 days. Methylene blue dye was completely decolourized within 6 days with using *Aspergillus* species (*Chithra et al*., 2013), within 21 day of fermentation with using *A. ochraceus* (Tisma *et al*., 2012), and 92.5% decolourization within 8 days (Ulkuye, 2013). *A. flavus* decolourised 98.86% of Congo red within 4 days (Sourav *et al*., 2011). The biosorption capacity of *A. flavus* was more effective than of *M. hiemalis*, where decolorization % with using *A. flavus* at 12 days was 69.72, 85.98 and 86.71% (Table 2) compared with *M. hiemalis*, where decolorization % was 40.11, 58.24 and 68.77 % (Table 3) at 125, 250 and 500 µg/100mL respectively**.** Gupta and Kumari (2012) reported that biosorption capacity of *Mucor mucedo* is enhanced by better growth rate and hence early decolorization is visualized.

Table 1: Decolorization of safranin with using growing , live and dead biomass (0.5g) of *A. flavus* and *M. hiemalis.*

Initial concentration (µg/100mL)	*A. flavus*						*M. hiemalis*					
	Decolorization %			Rest quantity (µg/100mL)			Decolorization %			Rest quantity (µg/100mL)		
	Growing	Live	Dead	Growing	Live	Dead	Growing	Live	Dead	Growing	Live	Dead
*Control	0.00	0.00	0.00	250	250	250	0.00	0.00	0.00	250	250	250
125	69.71	45.10	20.50	37.85	68.62	99.37	39.81	36.50	28.56	57.23	79.38	57.23
250	85.93	41.55	30.60	35.16	146.12	173.50	57.59	38.85	18.87	106.02	152.88	106.02
500	86.70	34.40	20.70	66.49	328.00	396.50	68.73	30.86	20.73	156.30	345.70	156.30

*Control, uninoculated growth medium containing 250µg safranin

Table 2: Decolorization % of safranin at different incubation period with using growing *A. flavus.*

Incubation period (Day)	Concentration of safranin (µg/100mL)								
	125			250			500		
	Decl. %	Rest quantity	Rest quantity %	Decl. %	Rest quantity	Rest quantity %	Decl. %	Rest quantity	Rest quantity %
2	11.21	118.91	95.12	10.33	226.36	90.54	10.02	450.20	90.04
4	28.12	109.68	87.74	15.92	225.12	90.04	13.30	423.51	84.70
6	45.46	80.31	64.24	35.53	166.25	66.50	39.76	221.63	44.32
8	65.43	45.47	36.37	83.67	54.68	21.87	85.54	81.35	16.27
10	69.71	37.85	30.28	85.93	35.16	14.06	86.70	66.49	13.29
12	69.72	37.88	30.30	85.98	35.19	14.36	86.71	66.43	13.28

Table 3: Decolorization % of safranin at different incubation period with using growing of *M. hiemalis.*

Incubation period (Day)	Concentration of safranin (µg/100mL)								
	125			250			500		
	Decl. %	Rest quantity	Rest quantity %	Decl. %	Rest quantity	Rest quantity %	Decl. %	Rest quantity	Rest quantity %
2	12.14	107.54	86.03	13.42	225.25	90.10	10.11	477.00	95.4
4	19.46	97.65	78.12	33.21	188.86	75.54	36.87	366.15	73.23
6	30.58	81.03	64.82	46.76	163.91	65.56	55.65	230.65	46.13
8	39.81	57.23	45.78	57.59	106.02	42.40	68.73	156.30	31.26
10	39.85	56.24	44.99	58.22	105.89	42.35	68.77	156.51	31.30
12	40.11	56.21	44.96	58.24	105.87	42.34	68.77	156.51	31.30

Toxicity of safranin:

There was no highest toxic effect of safranin on the growth of the two fungi (Fig.4) cultivated on agar medium but it showed retardation of growth on broth medium particularly with *M. hiemalis*. Where, the dry weight of *A. flavus* was 420.11 and 393.65 mg (Table 4), while dry weight of *M. hiemalis* was 266.08 and 160.50 mg at control and 500 µg safranin respectively, as it was confirmed by the study the diagnostic chraiteria of *A. flavus* and *M. hiemalis* under different safranin concentrations, where no changes were observed in

mycelia or sporangiospores/conidiospores except colour of hyphae as showed in Fig. (5). Also, it is clear that the pH of the culture extracts of the two fungi remained static throughout the study (Table 4). Increase in initial pH from 2 to 11, safranin sorption increased from 25% to 75%. This suggests possibility of electrostatic attraction between dye cation and biosorbent particle (Maurya and Mittal, 2011).

Table 4: Growth of of *A. flavus* and *M. hiemalis* at different concentrations of safranin with assessment of pH of metabolized medium.

Initial concentration (µg/100mL)	*A. flavus*		*M. hiemalis*	
	Growth (mg/100mL)	pH	Growth (mg/100mL)	pH
Control	420.04±0.06	8.82±0.02	266.17±0.29	8.16±0.14
125	399.01±0.02	8.33±0.29	180.40±0.35	8.90±0.17
250	395.15±0.26	8.15±0.13	178.44±0.38	8.79±0.25
500	393.43±0.38	8.17±0.29	160.33±0.29	8.84±0.21

Values are mean of three replicates ± standard deviation

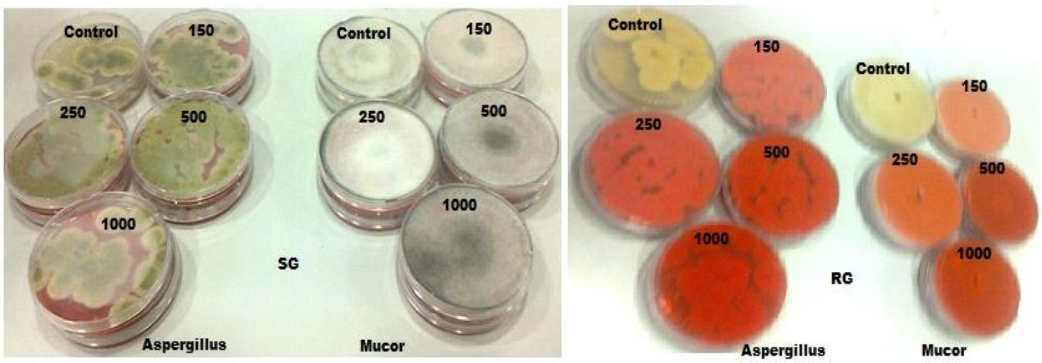

Fig. 4: Growth of *A. flavus* and *M. hiemalis* on medium supplemented with different concentration of safranin (150-1000µg/100ml). SG, surface of growth; RG, reverse of growth.

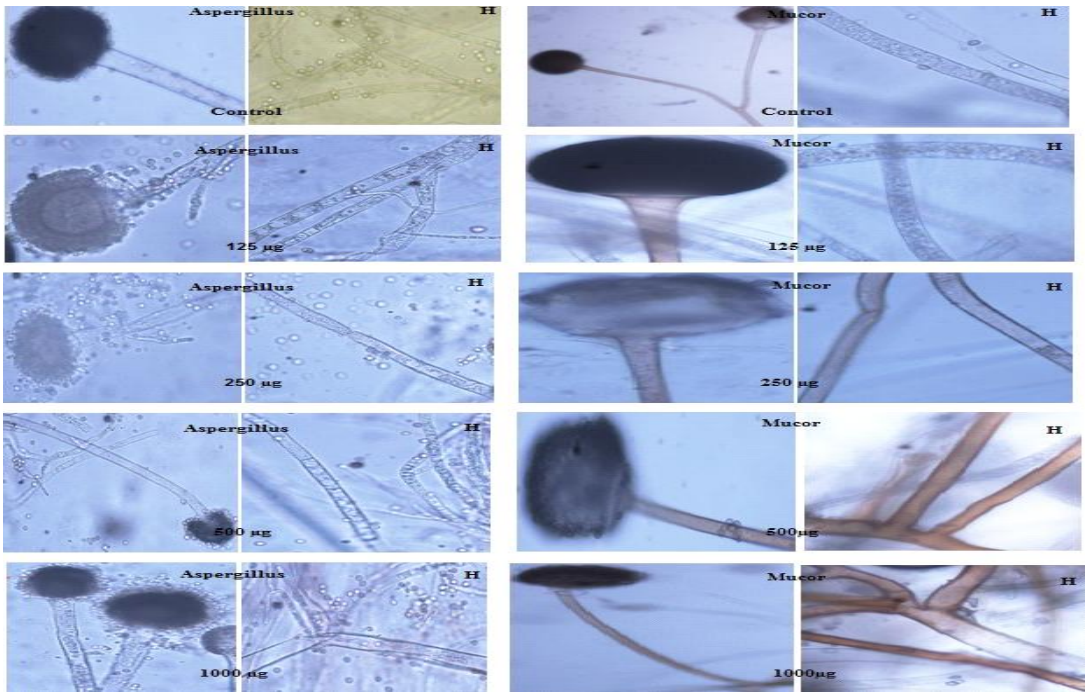

Fig. 5: Diagnostic charitеria of *A. flavus* and *M. hiemalis* at different concentration of safranin.

Impact of safranin on Metabolic changes* of *A. flavus* and *M. hiemalis:

Several studies with ligninolytic fungi demonstrated that the presence of fungal lignin peroxidase, manganese peroxidase and laccase seems to be correlated with their abilities to decolourise certain dyes (Glenn & Gold, 1983; Vyas and Molitores, 1995; Arora and Gill, 2000; Pant and Adholeya, 2007). Some fungi produce all three of them while other either one or two of them (Drista *et al*. 2007). Laccases are a diverse group of multi-copper proteins that catalyze the oxidation of a variety of aromatic compounds, including dyes. The enzymes, lignin peroxidase and laccase were estimated during the decolorization of safranin dye by *A. flavus*. Maximum decolorization % was 89.35 at safranin concentration 500 µg/100mL with the addition of lignin

compared with the decolorization% 85.93 without lignin addition. Production of these enzymes and decolorization % of safranin were increased with addition of lignin (Table 5). Our results correlated with the previous reports indicating that these enzymatic productions are responsible for the maximum decolourisation activities of safranin. *M. hiemalis* completely failed to secrete MnP and Laccase in medium growth containing high concentration (500 µg/100mL) of safranin, while the addition of lignin induce secretion these enzymes.

Degradation of dyes appeared to be due to the production of extracellular enzymes by *Mucor mucedo* (Gupta and Kumari, 2012) *A. flavus* (Deepak and Alok, 2009). Lignolytic system of *M. hiemalis* is not that much active as in *A. flavus*. But *M. hiemalis* showed also decolourisation activities indicating the role of mycelial biomass responsible for the decolourisation of the dye. Recently, Rania *et al*., (2013) revealed that *Emericella nidulans, Aspergillus fumigatus, Penicillium oxalicum and Humicola grisea* exhibited maximum potential for high lignin degradation and showed higher lignin peroxidase and laccase activities. The ligninolytic enzymatic activities had a great potential for decolorization of chemically different synthetic dyes safranin, crystal violet and malachite green. Tisma *et al*. (2012) stated that nonlignolytic strain *Aspergillus ochraceus* and its extracellular enzymes can be potential candidates for wastewater treatment of dye effluents. The present results are in agreement with Mtui and Nakamura, (2008) who found that Synthetic dyes led to high Lac, LiP, MnP activities.

Methyl orange decolorization efficiencies of *Fusarium*, *Trichoderma*, *Humicola*, *Aspergillus*, *Penicillium*, *Paecilomyces*, *Alternaria* and *Beauveria* sp. with live pellets were higher than autoclaved pellets. This result indicates that fungi are not successful biosorbents with pellets but perform decolorization with the help of enzymes that they produce (Seyis and Subasioglu, 2008). In fact, decolorization with microorganisms is carried out either with adsorption of the pigment on to mycelia or its enzymatic degradation (Nakajima-Kambe *et al*., 1999). Lignin peroxidase production (0.311 and 0.460 U/ml)) was recorded in *Mucor mucedo* during decolorization of crystal violet and malachite green respectively (Moturi and Singara-harya, 2009). These reports are supporting our results that though *A. favus and M. mucedo* did not show higher enzymatic activity, but still efficient in decolourisation dyes. Organic acids, mainly oxalic acid and citric acid are common metabolites produced by several fungi and their production is associated with the solubilisation of insoluble compounds containing metal. Different organic molecules, in particular di- and tri-carboxylic acids that do not belong to the matrix of the cell wall are excreted by fungal cells to chelate metal ions (Fomina *et al*., 2005). The obtained results showed that metabolic activities as well as fatty acids, organic acids and phenolic compounds of *A. flavus* and *M. hiemalis* were affected by safranin dye application (Table 6, 7& 8 and Fig.6&7). In our studies the presence of safranin dye in fungal growth medium suppressed the synthesis of certain fatty acids such as caprylic, linoleic, arachidic and behenic specially at high concentration of safranin (500µg/100mL). This fatty acids may be interfered with metabolic pathways of dye degradation. On the other hand few fatty acids (palmitic, oleic acids) increased in the concentration as a result of safranin application (Table 6). These fatty acids provide the strongest protective effect on cell membrane. According to the organic acid analysis results, it was found that the amount of fungal content of organic acids increased in the presence of safranin dye compared to the control (Table 7). On the other hand, certain organic acids such as gallic acid, acetic acid and citric acid syntheized in *M. hiemalis* only with the presence of safranin dye. Phenolic compounds concentration increased in fungal biomass cultivated in safranin containing medium (Table 8) indicated the vital role in safranin biodegradation.

Table 5: Decolorization of sarranin and enzymes detection of *A. flavus* and *M. hiemalis* cultivated on medium supplemented with lignin.

Safranin concentration (µg/100mL)	Lignin	*A. flavus*			*M. hiemalis*		
		Decl. %	**Lip (U/ml)	Laccase(U/ml)	Decl. %	**Lip (U/ml)	Laccase(U/ml)
*Control	-	0.00	0.00±0.00	0.00±0.0	0.00	0.0±0.0	0.0±0.0
	+	0.00	0.45±0.02	3.98±0.05	0.00	0.35±0.05	3.78±0.06
125	-	69.71	0.20±0.08	1.33±0.06	35.81	0.12±0.03	1.25±0.05
	+	73.50	0.47±0.03	4.35±0.02	39.95	0.39±0.02	4.22±0.08
250	-	85.93	0.25±0.05	1.25±0.02	57.59	0.32±0.07	1.20±0.02
	+	89.35	0.59±0.06	5.25±0.07	60.50	0.64±0.02	4.20±0.02
500	-	86.70	0.26±0.05	1.26±0.01	68.73	0.11±0.06	1.19±0.06
	+	87.50	0.62±0.06	3.60±0.02	68.00	0.38±0.06	2.71±0.03

*Control, safranin less inoculated growth medium with (+) or without (-) Lignin, **Lignin peroxidase (Lip), Values are mean of three replicates ± standard deviation

Table 6: Fatty acids percentage (%) of *A. flavus* and *M. hiemalis* Cultivated on growth medium at different concentrations of safranin(µg/100mL).

Fatty acid		Fatty acid percentage (%) at different concentrations of safranin (µg/100mL)					
		A. flavus			*M. hiemalis*		
		*Control	125	500	*Control	125	500
Caprylic	C8	3.44	0.0	0.0	2.12	0.0	0.0
Capric	C10	2.5	1.20	0.0	0.42	0.0	0.0
Lauric	C12	2.22	7.12	0.0	3.74	4.26	1.34

Tridecanoic	C13	7.88	2.31	0.0	3.25	1.98	0.45
Myristic	C14	5.43	14.74	0.0	10.86	15.73	9.81
Pentadecanoic	C15	2.0	0.32	0.0	1.80	0.56	3.28
Palmitic	C16	29.7	42.35	53.84	26.8	24.38	37.06
Palmitoleic	C16:1	2.81	0.0	0.0	2.10	3.97	5.88
Heptadecanoic	C17	4.92	4.12	0.0	3.78	4.78	3.12
Stearic	C18	9.83	3.86	4.89	15.47	13.2	10.86
Oleic	C18:1	19.59	23.98	41.27	22.3	31.02	28.2
Linoleic	C18:2	6.36	0.0	0.0	7.82	0.12	0.0
Arachidic	C20	3.24	0.0	0.0	0.0	0.0	0.0
Behenic	C22	0.08	0.0	0.0	0.0	0.0	0.0
Total of Saturated Fatty acid		71.24	76.02	58.73	67.78	64.89	65.92
Total of Unsaturated Fatty acid		28.76	23.98	41.27	32.22	35.11	34.08

*Control, inoculated growth medium without safranin

Table 7: Organic acids percentage (%) of *A. flavus* and *M. hiemalis* cultivated on growth medium at different concentrations of safranin(µg/100mL)

Organic acid	Organic acid concentration (mg/ml) at different concentrations of safranin(µg/100mL)					
	A. flavus			*M. hiemalis*		
	*Control	125	500	*Control	125	500
Citric acid	23.47	155.49	15.223	0.0	130.56	0.0
Oxalic acid	0.0	0.0	50.64	0.0	0.0	0.0
Fumaric acid	14.12	0.0	0.0	0.0	0.0	0.0
Gallic acid	0.0	53.20	0.0	0.0	26.36	0.0
Acetic acid	0.0	0.0	0.0	0.0	1.2	0.0

*Control, inoculated growth medium without safranin

Table 8: Phenolic compounds concentration (mg/ml) of *A. flavus* and *M. hiemalis* cultivated on growth medium at 125 and 500 µg/100mL of safranin.

Phenolic compounds	Phenolic compounds concentration (mg/ml) at different concentrations of safranin (µg/100mL)					
	A. flavus			*M. hiemalis*		
	*Control	125	500	*Control	125	500
Ferulic acid	0.0	0.0	0.0	0.0	25	126.22
Caffieic acid	6.0	45.28	0.0	0.0	6.0	0.0
Catecol	0.0	42.66	0.0	0.0	18	25.34
Coumaric acid	5.87	3.4	0.0	0.0	0.0	2.36

*Control, inoculated growth medium without safranin

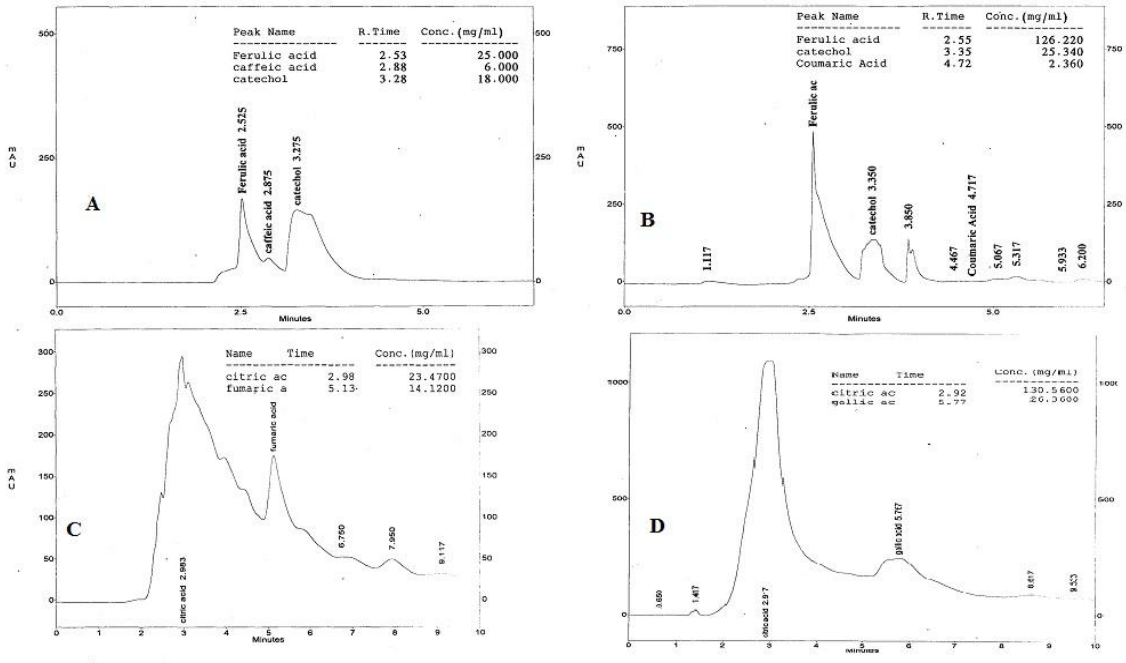

Fig. 6: HPLC chromatogram of detected phenolic and organic acids of *M. hiemalis* and *A. flavus* (A, phenolic compounds of *M. hiemalis* at 125µg/100mL safranin, B at 500µg/100mL safranin; C, Organic acids of *A. flavus* at Control; D Organic acid of *M. hiemalis* 125µg/100mL safranin.

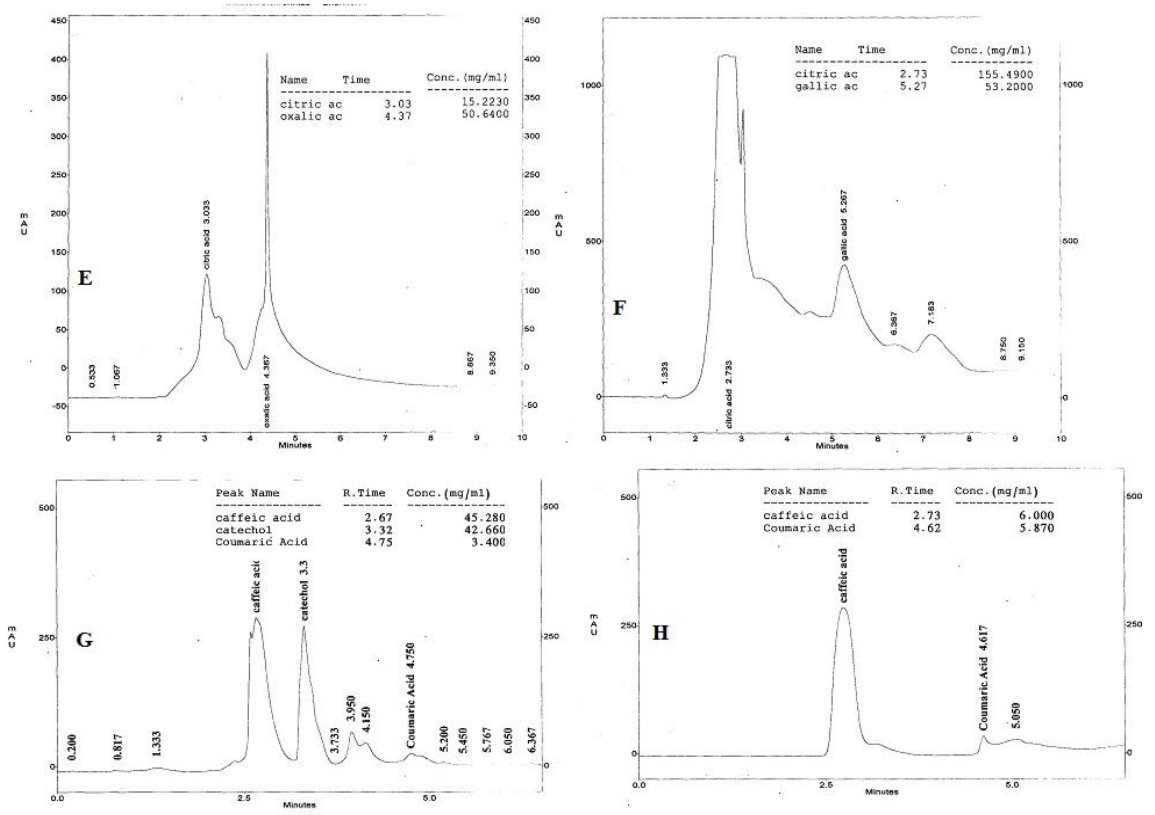

Fig. 7: HPLC chromatogram of detected phenolic and organic acids of *M. hiemalis* and *A. flavus* (E, Organic acids of *A. flavus* 500µg/100mL and F, 125µg/100mL safranin; G, phenolic compounds of *A. flavus* at 125µg/100mL;H, phenolic compounds of *A. flavus* at control.

Conclusion:

According to conditions above described, we can state that *A. flavus* and *M. hiemalis* will contribute to the decolorization and degradation of effluents containing safranin dye and their action will reduce the pollutant discharge and toxicity related to the presence of dyes.

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