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First report on implementation of response surface methodology for the biodegradation of textile industrial effluents by *Coniophora puteana* IEBL-1

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Abstract: The current study was aimed to evaluate the industrial effluents biodegradation potential of an indigenous microorganism which reduced water pollution caused by these effluents. In the present study biodegradation of three textile industrial effluents was performed with locally isolated brown rot fungi named *Coniophora puteana* IEBL-1. Response Surface Methodology (RSM) was employed under Box Bhenken Design (BBD) for the optimization of physical and nutritional parameters for maximum biodegradation. Quality of treated effluents was checked by study of BOD, COD and analysis through HPLC. Three ligninolytic enzymes named lignin peroxidase, manganese peroxidase and laccase were also studied during the biodegradation process. The results showed that there was more than 85% biodegradation achieved for all three effluents with decrease in Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) below the recommended values for industrial effluent i.e. 80 mg/L for BOD and 220 mg/L for COD after optimization of nutritional parameters in the second stage. Analysis of samples through HPLC revealed the formation of less toxic diphenylamine, 3-methyldiphenylamine and N-methylaniline after treatment. The ligninolytic enzymes assays confirmed the role of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase in biodegradation process. Lignin peroxidase with higher activity has more contribution in biodegradation of effluents under study. It can be concluded through the results that *Coniophora buteana* IEBL-1 is a potential fungus for the treatment of industrial effluents.

Introduction

Currently almost all the textile industries use synthetic dyes as coloring agents because these give more colors and are long lasting. Sufficient amount of these dyes entered into water bodies in the form of effluents and is responsible for water pollution (Iqbal and Asgher 2013). Most of the synthetic dyes are toxic and may cause certain diseases to human beings after entering into food chain from water bodies and are constant source of environmental pollution (Arora 2014, Kyzioł-Komosinska et al. 2011). Reduction in environmental pollution is a great challenge for human beings and it is also a main focus of the scientists (Demirci et al. 2011).

Scientists are extensively involved in the research on the development of new techniques for the degradation of dyes and other coloring agents, i.e. pigments, to reduce pollution and health risk to human beings. The physical and chemical techniques, which are used around the world in industrial sector, are non-economic, they generate other toxic pollutants and are non-feasible for most of industrialists (Pavko 2011, Shakir et al. 2010, Singh 2006). Compared to physical and chemical methods of dyes and effluent degradation, biological degradation would be socio-economic, feasible for all users and usually completely mineralize the dyes (Ali et al. 2010, Moosvi et al. 2005, Pavko 2011). Biological treatment has the ability to efficiently reduce pollutants in the effluents by biodegradation (Bawlec et al. 2016).

Biodegradation involves the use of microorganisms for degradation/decolorization of dyes in the effluent (Hassan et al. 2013). The most commonly used microorganisms are bacteria and fungi. Fungi are the first choice of a researcher because of their versatile nature of growth in harsh conditions with minimal requirements. Brown rot fungi, a group of wood decaying fungi, produce active extracellular ligninolytic enzymes and have the ability to modify plant cell wall (Ali et al. 2010, Piontek et al. 2001). They may be a potential group of fungi for the structurally resembled synthetic textile dyes in effluent (Gao et al. 2010, Sanchez 2009). There are a few studies on role of brown rot fungi in the biodegradation of dyes and more attention is required to explore the potential of this group of fungi. There are some studies that highlight the role of brown rot fungi and ligninolytic enzymes produced by these fungi in the biodegradation/decolorization of dyes, which are present in effluents (Ali et al. 2010, Ambrosio and Campos 2004, Samuthi and Manju 2000, Zhang et al. 2007).

The objectives of the current study were to explore the biodegradation potential of industrial effluents by *C. puteana* IEBL-1, brown rot fungi, under optimum conditions. It also involved the study of ligninolytic enzymes involved in biodegradation process and finally analysis of treated effluents by HPLC.

Materials and methods

Chemicals and reagent

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS, manganese sulfate and veratryl alcohol were of Sigma and purchased from local supplier. Ligninolytic enzymes (laccase, lignin peroxidase and manganese peroxidase) were produced from *Coniophora puteana* IEBL-1. Untreated industrial effluents from the following industries: Mujahid Textile industry (λmax = 513), Sitara Textile industry (λmax = 642) and Five Star Textile industry (λmax = 518) were collected from industrial units. All other chemicals used for the enzymatic assay and media preparation were of analytical grade, whereas those for HPLC analysis were of HPLC grade and purchased from local chemical supplier.

Fungal collection and isolation

Coniophora puteana IEBL-1 was obtained from Industrial and Environmental Biotechnology (IEBL) Laboratory, University Institute of Biochemistry and Biotechnology, PMAS-Arid Agriculture University Rawalpindi. It was isolated on malt extract agar media having pH 5.5 (Asgher et al. 2012). Broth culture (inoculum) was prepared for subsequent use in biodegradation process. Malt Extract inoculum media was used during this study, it composed of malt extract 20 g/L, glucose 20 g/L, peptone 3 g/L and distilled water. The fungal spores were shifted from pure culture into inoculum media and put on shaking incubator for 3–4 days. The number of spores was monitored each day with biomass monitor (ABER-220 model) and inoculum with 10^7–10^9 spores/mL was used in biodegradation process.

Biodegradation of textile effluents

The biodegradation of textile effluents by *C. puteana* IEBL-1 was initially observed for 8 days. The biodegradation process was then optimized at two stages employing Response Surface Methodology (RSM) under Box Bhenken Design (BBD) (Asgher et al. 2012). At each stage five parameters were optimized simultaneously and experiments were designed using JMP software. The experiments were performed with 90 mL effluent in 250 mL flasks which were autoclaved, all conditions mentioned in table 1, and put on shaking incubator at 120 rpm for mentioned time period. To check the biodegradation the sample was filtered and 2 mL of the mixture was withdrawn. It was centrifuged at 10,000 rpm for 15 mins before determination of % biodegradation and performing ligninilytic enzymes assay. To check the biodegradation, absorbance was noted before and after the process at λmax of each effluent. The efficiency of biodegradation was also monitored by checking decrease in Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). The biodegradation (%) was calculated by the following formula:

$$\%\ Biodegradation = \frac{(A_{ini} - A_{fin}) \cdot 100}{A_{ini}}$$

Where:
A_{ini} – initial absorbance of effluent before incubation,
A_{fin} – final absorbance of effluent after incubation.

Optimization of physical parameters

The fungal growth and biodegradation by fungi are associated with certain factors, such as pH, temperature, time period, effluent conc. and fungal inoculum size (Asgher et al. 2012, Pavko 2011, Singh 2006). These parameters were optimized simultaneously with 46 experimental combinations having six central points (Table 1).

Optimization of nutritional parameters

The addition of readily available various carbon and nitrogen sources may increase fungal growth, enzyme secretion and ultimately the biodegradation of dyes in effluent. To monitor this, five carbon (glucose and fructose) and nitrogen (ammonium sulfate, ammonium nitrate and ammonia) sources were added into the biodegradation mixture. There were 46 combinations of experiments with 6 central points, designed under RSM and run in triplicate (Table 2).

Quality analysis of treated industrial effluents

To monitor the efficacy of biodegradation process and to check the quality of treated effluents Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) were measured.

Determination of BOD

The BOD of each industrial effluent was measured for 5 days by the method as described by Greenberg et al. 1985. The effluent under observation was packed in air tight bottle for 5 days and dissolved oxygen was measured initially as well as at last day. The oxygen taken during these days was oxygen used by microorganisms for biodegradation of material.

$$BOD_5 = Initial\ dissolved\ oxygen - Final\ dissolved\ oxygen\ (after\ 5\ days)$$

The dissolved oxygen was measured by the Winkler method which involved the oxidation of Mn(II) ion from $MnSO_4$ into

Table 1. Experimental design for the optimization of physical parameters during the biodegradation of effluents

No.	pH	Inoculum size (mL)	Days	Temp. (ºC)	Effluent Conc. (%)	No.	pH	Inoculum size (mL)	Days	Temp. (ºC)	Effluent Conc. (%)
1.	8	6	2	32.5	0.03	24.	6	6	5	32.5	0.03
2.	4	6	2	32.5	0.03	25.	6	6	5	32.5	0.03
3.	8	6	5	32.5	0.01	26.	6	6	2	32.5	0.01
4.	4	6	5	40	0.03	27.	8	6	5	32.5	0.05
5.	4	6	5	32.5	0.01	28.	6	6	2	40	0.03
6.	6	2	8	32.5	0.03	29.	4	6	5	32.5	0.05
7.	4	10	5	32.5	0.03	30.	6	6	8	32.5	0.01
8.	6	6	5	40	0.05	31.	6	10	5	32.5	0.01
9.	6	6	5	32.5	0.03	32.	6	6	8	25	0.03
10.	4	6	5	25	0.03	33.	8	6	5	40	0.03
11.	6	6	2	25	0.03	34.	6	10	5	40	0.03
12.	6	2	5	32.5	0.01	35.	6	2	5	25	0.03
13.	8	6	8	32.5	0.03	36.	6	10	5	25	0.03
14.	6	6	2	32.5	0.05	37.	6	6	8	40	0.03
15.	4	2	5	32.5	0.03	38.	6	10	2	32.5	0.03
16.	6	10	8	32.5	0.03	39.	6	2	5	32.5	0.05
17.	6	10	5	32.5	0.05	40.	6	6	5	32.5	0.03
18.	8	2	5	32.5	0.03	41.	6	6	5	25	0.05
19.	6	2	5	40	0.03	42.	6	2	2	32.5	0.03
20.	6	6	5	25	0.01	43.	6	6	5	32.5	0.03
21.	6	6	5	32.5	0.03	44.	6	6	5	40	0.01
22.	8	6	5	25	0.03	45.	6	6	8	32.5	0.05
23.	4	6	8	32.5	0.03	46.	8	10	5	32.5	0.03

Table 2. Experimental design for the optimization of nutritional parameters during the biodegradation of effluents

No.	Glucose (%)	Fructose (%)	Amm. nitrate (%)	Amm. sulfate (%)	Ammonia (%)	No.	Glucose (%)	Fructose (%)	Amm. nitrate (%)	Amm. sulfate (%)	Ammonia (%)
1.	1.50	1.50	1.50	1.50	1.50	24.	1.00	2.00	1.00	1.00	2.00
2.	2.00	2.00	2.00	2.00	2.00	25.	2.00	1.00	1.00	2.00	1.00
3.	1.00	1.00	1.00	1.00	1.00	26.	2.00	1.00	2.00	1.00	2.00
4.	1.00	1.00	1.00	1.00	2.00	27.	1.00	1.00	1.00	1.00	1.00
5.	1.00	1.00	1.00	2.00	2.00	28.	2.00	1.00	1.00	1.00	2.00
6.	1.00	1.00	2.00	2.00	2.00	29.	1.00	2.00	1.00	2.00	1.00
7.	1.00	2.00	2.00	2.00	2.00	30.	2.00	1.00	2.00	2.00	1.00
8.	2.00	1.00	1.00	1.00	1.00	31.	1.00	2.00	2.00	2.00	1.00
9.	2.00	2.00	1.00	1.00	1.00	32.	2.00	2.00	1.00	2.00	2.00
10.	2.00	2.00	2.00	1.00	1.00	33.	1.00	2.00	2.00	1.00	2.00
11.	2.00	2.00	2.00	2.00	1.00	34.	2.50	1.50	1.50	1.50	1.50
12.	2.00	1.00	2.00	1.00	1.00	35.	1.50	1.50	1.50	1.50	0.50
13.	2.00	2.00	1.00	1.00	2.00	36.	1.50	1.50	1.50	2.50	1.50
14.	1.00	2.00	1.00	2.00	2.00	37.	1.50	1.50	1.50	0.50	1.50
15.	1.00	1.00	2.00	1.00	2.00	38.	0.50	1.50	1.50	1.50	1.50
16.	2.00	1.00	1.00	2.00	2.00	39.	1.50	1.50	0.50	1.50	1.50
17.	2.00	2.00	2.00	1.00	2.00	40.	1.50	2.50	1.50	1.50	1.50
18.	1.00	1.00	1.00	2.00	1.00	41.	1.50	1.50	2.50	1.50	1.50
19.	1.00	2.00	2.00	1.00	1.00	42.	1.50	1.50	1.50	1.50	2.50
20.	2.00	1.00	2.00	2.00	2.00	43.	1.50	0.50	1.50	1.50	1.50
21.	1.00	1.00	2.00	2.00	2.00	44.	1.00	1.50	1.50	0.50	1.00
22.	2.00	2.00	1.00	1.00	1.00	45.	1.50	1.00	1.00	1.50	0.50
23.	2.00	2.00	1.00	2.00	1.00	46.	0.50	0.50	1.00	1.00	1.50

Mn(IV) ion by dissolved oxygen. The Mn_2O_3 dissolved in sulphuric acid and produced Mn^{3+} ion, which reacted with added iodide to produce iodine (I_2). The amount of I_2 was then determined by titrating against sodium thiosulfate and starch as an indicator (Greenberg et al. 1985).

Determination of COD

The COD determines the total oxygen required to oxidize organic matter in the waste water and effluent. It was also measured by the method described by Greenberg et al. 1985. The determination of COD involves the oxidation of organic material in the effluent with strong oxidizing solution/digestion solution ($K_2Cr_2O_7$, $HgSO_4$ and (98%) H_2SO_4). A catalyst solution was used to enhance oxidation process which comprises of Ag_2SO_4 in H_2SO_4. To determine the value of COD in effluents, 1.5 mL of digestion mixture was placed in closed vial along with 3.5 mL of catalyst solution and 2.5 mL of effluent at 150ºC in oven for 2 hrs. The values of COD were estimated by taking absorbance at 600 nm. Blank consisted of all solutions except effluents while potassium hydrogen phthalate (KHP) was used as standard (Greenberg et al. 1985). The value of COD for effluents was calculated by the following formula;

$$COD = Standard\ factor \cdot Absorbance\ at\ 600\ nm$$

Where:
Standard factor – concentration of standard/absorbance of standard.

Study of ligninolytic enzymes

Three ligninolytic enzymes expected to be secreted by *C. puteana* IEBL-1 during biodegradation of effluents were studied during each experiment. A change in the activities with biodegradation will give an idea about the involvement of these enzymes in the process.

Study of lignin peroxidase (LiP)

Lignin peroxidase activity assay was performed by the method of Tien and Kirk 1984 by using veratryl alcohol (3,4-dimethoxybenzylalcohol) as substrate. The reaction mixture contained 1 mL of tartarate buffer (100 mM, pH=4), 1 mL veratryl alcohol and 500 µL H_2O_2 (0.2 M). The reaction started after the addition of 100 µL of biodegradation mixture as enzyme sample. Blank contained distilled H_2O instead of enzyme sample. The activity was measured by taking absorbance after the interval of 10 mins at 310 nm and was calculated by using the following formula.

$$A = \epsilon_{310} \cdot c \cdot l$$
$$\epsilon_{310} = 9{,}300\ M^{-1} \cdot cm^{-1}$$

Where:
Є – molar extinction coefficient,
c – concentration,
A – absorbance,
l – path length.

Study of manganese peroxidase (MnP)

Manganese peroxidase activity assay was performed by the method described by Wariishi et al. 1992. Manganese sulfate was used as substrate ($MnSO_4$) and H_2O_2 as an oxidizing agent. The reaction mixture consisted of 1 mL of $MnSO_4$, 1 mL sodium melonate buffer (50 mM, pH=4.5) and 500 µL H_2O_2. The addition of 100 µL of enzyme solution started the reaction The complex products were monitored spectrophotometrically at 270 nm initially and after 10-min interval. The activity of MnP was calculated by using the following formula.

$$A = \epsilon_{270} \cdot c \cdot l$$
$$\epsilon_{270} = 11{,}590\ M^{-1} \cdot cm^{-1}$$

Study of laccase

Laccase activity assay was performed by the method reported by Wolfenden and Wilson 1982 by using ABTS as substrate. Laccase activity assay was performed by mixing 1 mL ABTS solution in 1 mL sodium melonate buffer (50 mM, pH=4.5) and 100 µL enzyme sample. The absorbance was noted at 410 nm initially and after the interval of 10 mins. The difference in absorbance was used to calculate the laccase activity by using the following formula.

$$A = \epsilon_{436} \cdot c \cdot l$$
$$\epsilon_{436} = 36{,}000\ M^{-1} \cdot cm^{-1}$$

HPLC analysis of the industrial effluent

HPLC analysis of the untreated and treated effluent was performed to monitor the products during biodegradation of textile industrial effluent. The HPLC was performed on SHIMAZDU LC-20AT model having C-18 column of 25 mm length with internal diameter of 4.6 mm. The analysis was performed in isocratic and reverse phase mode at 1 mL/min. flow rate. The solvent system was acetonitrile:water with (60:40) ratio and 20 µL of each sample was injected for analysis (Çelik et al. 2012, Zhao and Hardin 2007).

Results and discussion

It was reported earlier by Mahmood et al. 2017 that *C. puteana* IEBL-1 has a very good ability of biodegradation of various disperse textile dyes during screening of different brown rot fungi for their potential. In continuation of that study, followed by successful optimization and enhancement of biodegradation of textile dyes by this fungus, the potential was tested on real industrial effluents. The classical RSM was employed under Box Bhenken Design.

Textile industries utilized high contents of coloring agents for the colorization of wide variety of textile products. The amount of dyes applied on fibers did not fix and 15–20% released as effluent. Some dyes are reported to have carcinogenic effects in human body. Brown rot fungi have the potential of complete degradation of these dyes into non-toxic form due to their non-specific ligninolytic enzyme system (Asgher et al. 2013, Awasthi and Prakash 2014, Kanmani et al. 2011).

Effect of physical parameters on biodegradation of textile effluents

C. puteana IEBL-1 showed a very good biodegradation activity on textile effluents under study. The results obtained from experiments in Table 1 were run on JMP software to find the most suitable experimental conditions for maximum biodegradation.

The % biodegradation suggested by software from the results was verified by performing experiment again at these conditions. There was 83.02±1.43% biodegradation of Mujahid Textile (MT) industrial effluent at pH 5.74, inoculum size 6.07 mL, time period 7.83 days, temperature 26.77ºC and dye concentration 0.02% (Fig. 1). Very low P value (P<0.001), high F ratio (28.58) and high R^2 (R^2=96.14%) indicated the significant effect of parameters optimized on biodegradation of MT industrial effluent (Selvakumar et al. 2013, Vijayalakshmidevi and Muthukumar 2015). There was 75.02±1.45% biodegradation achieved for Five Star Textile (FST) industrial effluent by *C. puteana* IEBL-1 at pH 5.85, inoculum size 5.79 mL, time period 7.99 days, temperature 26.58ºC and dye concentration 0.027%. The study of 3D response surface graphs showed that biodegradation achieved was the combinational effect of all the parameters under study (Fig. 2). The statistical analysis indicated a significant effect of these parameters on biodegradation, very low P value (P=0.0001), high F ratio (26.65) and R^2 value (R^2=96%). In the case of Sitara Textile (ST) industrial effluent there was 84±1.32% biodegradation achieved at pH 5.76, inoculum size 6.09 mL, time period 7.90 days, temperature 26.68ºC and dye conc. 0.029% (Joshi et al. 2012). Analysis of 3D response surface graphs obtained from JMP software showed positive interaction between selected parameters to give maximum results (Fig. 3). Significance of these results was indicated by very low P value (P=0.001), high F ratio (27.7) and R^2 (R^2=96%) (Demirci et al. 2011, Hassan et al. 2013, Singh et al. 2015). The samples treated at the above mentioned most suitable conditions were further studied for the determination of BOD and COD, in order to monitor the quality of treated samples.

Effect of carbon and nitrogen sources on biodegradation of textile effluents

There was a significant increase in biodegradation of all three textile effluents when treated with *C. puteana* IEBL-1 after adding carbon and nitrogen sources. The biodegradation of Mujahid Textile effluent increased from 83.02±1.43% to 93.21±1.73% in the presence of 1% of each of the five nutrients under study. There was 11.79% increase in biodegradation of FST industrial effluent (75.02±1.45% to 86.81±1.81%) and 11.03% increase in ST industrial effluent (84±1.32% to 95.03±1.63%) when treated with *C. puteana* IEBL-1 at optimized conditions and in the presence of 1% of each of the nutrients (Table 3). The presence of all nutrients i.e. glucose, fructose, ammonia, ammonium sulfate and ammonium nitrate with ratio 1:1 (1% each) was found suitable for the biodegradation of all three industrial effluents. Carbon source up to 2% and nitrogen sources (ammonium sulfate and ammonium nitrate) up to 1.5% have positive effect on biodegradation. The addition of more than 1.0% of ammonia negatively affects the biodegradation process, possibly due to the un-stability of enzymes involved in biodegradation (Kabra et al. 2013, Mahmood et al. 2015, Vijayalakshmidevi and Muthukumar 2015). The samples treated after the addition of 1% of each of the nutrients were further studied for the determination of BOD and COD for quality analysis.

Study of ligninolytic enzymes during optimization of biodegradation

The biodegradation of all three textile industrial effluents was increased by *C. puteana* IEBL-1 after both optimization steps. The activities of all three ligninolytic enzymes i.e. lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase, were also increased with increasing biodegradation, which showed the involvement of these enzymes in the process. After optimization of physical parameters, maximum ligninolytic activities were 908.6±2.1 $U{\cdot}mL^{-1}min^{-1}$, 680.45±3.21 $U{\cdot}mL^{-1}min^{-1}$ and 377.65±2.13 $U{\cdot}mL^{-1}min^{-1}$ for LiP, MnP and laccase respectively. These activities further increased after optimization of carbon and nitrogen sources along with an increase in biodegradation of the dyestuff. The activities after the second step of optimization were 1020.4±4.2 $U{\cdot}mL^{-1}min^{-1}$, 735.55±4.89 $U{\cdot}mL^{-1}min^{-1}$ and 443.5±3.21 $U{\cdot}mL^{-1}min^{-1}$ for LiP, MnP and laccase, respectively. These findings suggest the role of ligninolytic enzymes of *C. puteana* IEBL-1 in the biodegradation of the dyestuff. Higher activity of LiP indicated that this enzyme has a more active role in the biodegradation of industrial effluents under study (Kumar et al. 2011, Mahmood et al. 2015).

The ligninolytic enzymes, especially MnP, involved in the oxidation of phenolic substrates which are part of dyes in the effluents. The oxidized products of phenolics dyes act as substrate of other enzymes (Kunjadia et al. 2016). Kunjadia et al. in 2016 reported the biodegradation of dyes by ligninolytic enzymes from fungi; the biodegradation enhanced with increased enzymes activities. The Azo dyes are decolorized by ligninolytic enzymes produced by fungi, when mixture is supplemented with optimized conditions. This decolorization is achieved by oxidative degradation of dyes contents in the mixture by ligninolytic enzymes including LiP, MnP and laccase (Sing et al. 2017).

Determination of BOD and COD of bio-treated textile effluents

Due to the biodegradation of industrial effluents their quality would improve which can be monitored by studying their Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). Treated industrial effluents with BOD and COD within recommended values could be utilized for irrigation and other useful processes without much negative effects (Elkassas and Mohamed 2014).

For textile industrial effluents the permitted values are 250 mg/L for COD and 80 mg/L for BOD. The initial BOD of MT industrial effluent was 358.27 mg/L and COD was 1032.1 mg/L. There was 90.22% (35.1±0.7 mg/L) reduction in BOD and 82.94% (176.1±1.32 mg/L) reduction in COD of MT effluent after both steps of optimization. Initial values of BOD and COD of FST industrial effluent were 347.2 mg/L and 1176.2 mg/L respectively. There was 91.06% (31.2±0.72 mg/L) and 85.96% (165.5±2.01 mg/L) reduction in BOD and COD of FST effluent achieved after two-step optimization of the process. There were very high initial values of BOD (412.2 mg/L) and COD (1237.6 mg/L) calculated for ST industrial effluents. There was 93.90% (25.08±0.61 mg/L) decrease in BOD and 84.80% (188.2±1.91 mg/L) in COD after optimization of biodegradation in two steps. The process of biodegradation reduced the values within recommended values which confirmed the significance of this process. High BOD and COD made aquatic life difficult while reduction in these values made water suitable for aquatic life (Elkassas and Mohamed 2014, Mtui and Nakamura 2008, Porwal et al. 2015).

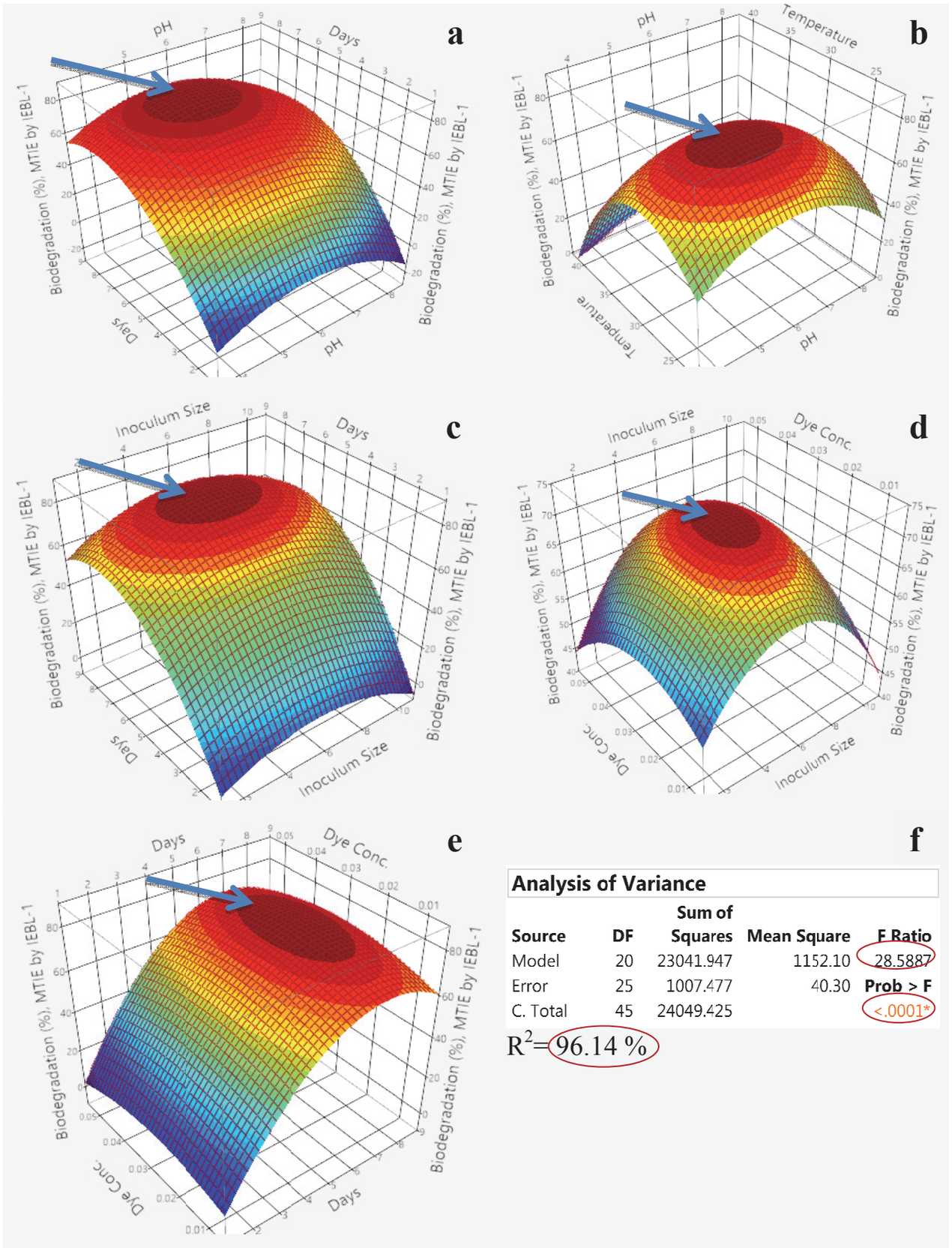
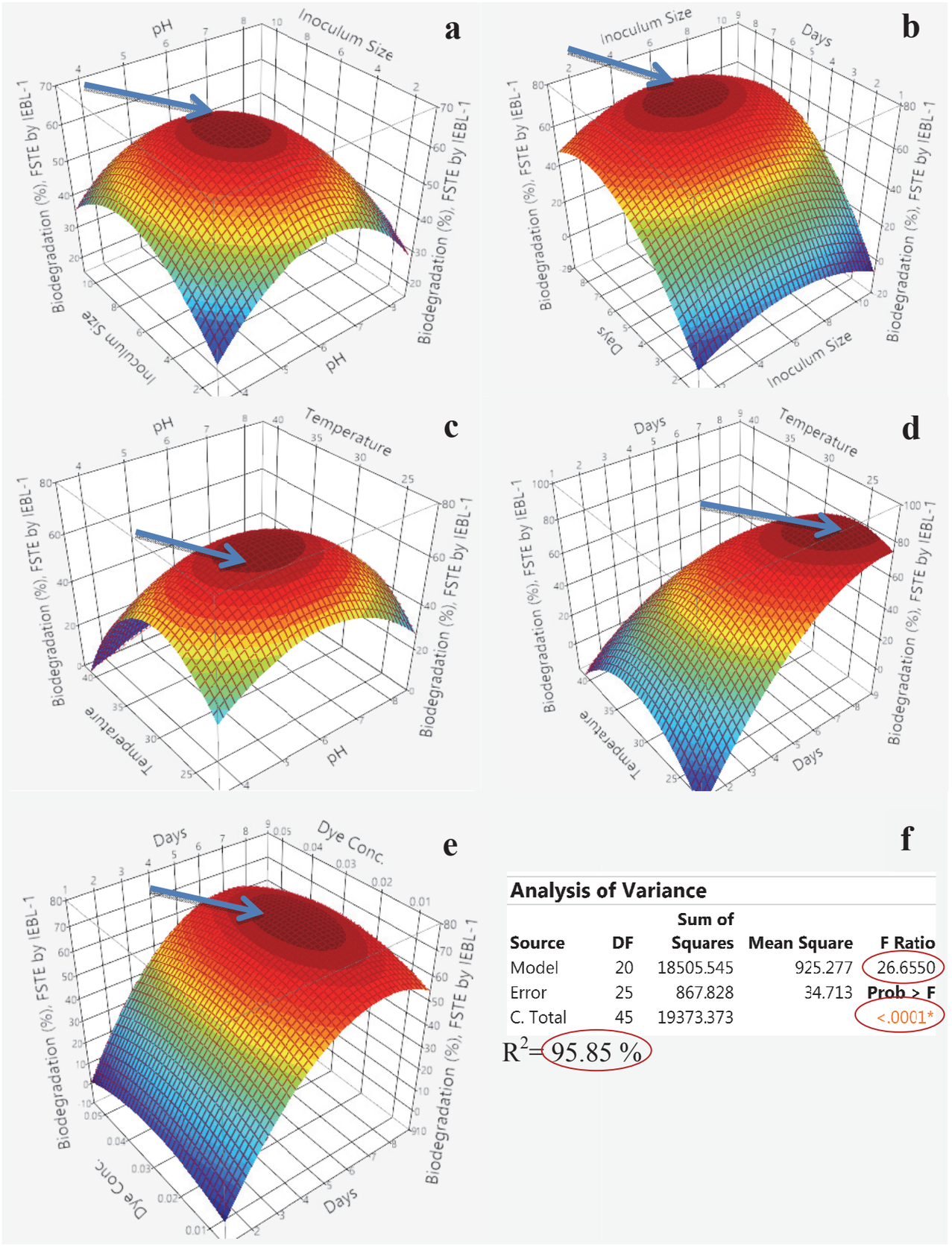


Fig. 1. Response surface 3D graphs showing interaction between various parameters during the biodegradation of Mujahid Textile (MT) industrial effluent by *Coniophora puteana* IEBL-1, graphs represent positive interaction between (a) time period and pH (b) temperature and pH (c) time period and inoculum size (d) dye concentration and inoculum size (e) dye concentration and time period (f) statistical analysis of results showing significant effect of parameters on biodegradation



f

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	20	18505.545	925.277	26.6550
Error	25	867.828	34.713	Prob > F
C. Total	45	19373.373		<.0001*

R^2= 95.85 %

Fig. 2. Response surface 3D graphs showing interaction between various parameters during the biodegradation of Five Star Textile (FST) industrial effluent by *Coniophora puteana* IEBL-1, graphs represent positive interaction between (a) inoculum size and pH (b) time period and inoculum size (c) temperature and pH (d) temperature and time period (e) dye concentration and time period (f) statistical analysis of results showing significant effect of parameters on biodegradation

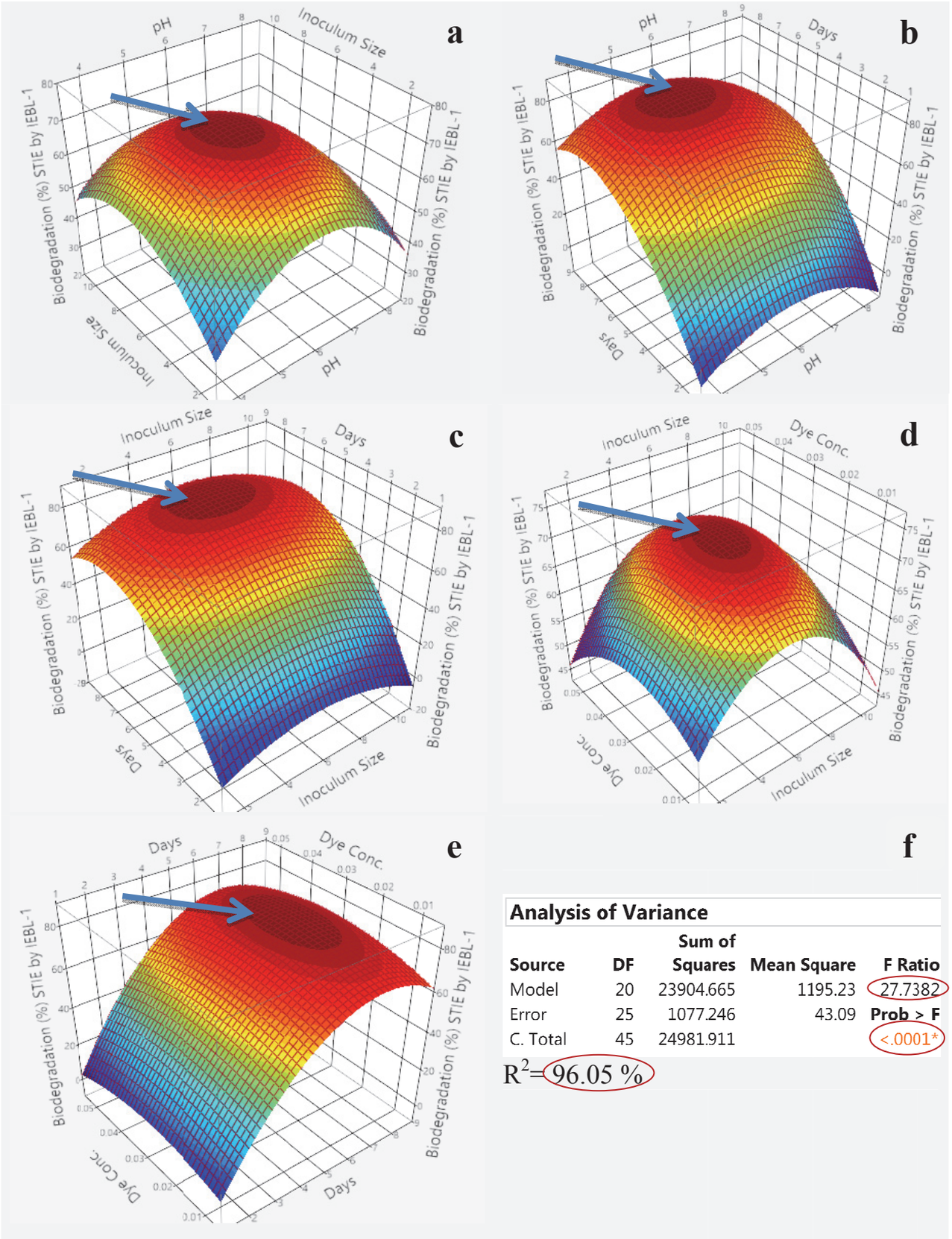


Fig. 3. Response surface 3D graphs showing interaction between various parameters during the biodegradation of Sitara Textile (ST) industrial effluent by *Coniophora puteana* IEBL-1, graphs represent positive interaction between (a) inoculum size and pH (b) time period and pH (c) time period and inoculum size (d) dye concentration and inoculum size (e) dye concentration and time period (f) statistical analysis of results showing significant effect of parameters on biodegradation

Luczkiewicz et al. in 2011, reported the reduction in COD, BOD and Total Suspended Solid (TSS) of wastewater treated with various physical techniques but the ratio of reduction (a difference between initial value and final value) was low compared to our results during current study. The significant reduction in the values of BOD and COD of effluents suggested that *C. puteana* IEBL-1 is very a efficient brown rot fungus that completely mineralizes the maximum amount of colorant in the effluents. The biodegradation and mineralization of dyes in effluent lead to the reduction of BOD and COD and improve the quality of effluent.

HPLC analysis of bio-treated industrial effluents

Textile effluents contain mixture of complex dyes, which are toxic in nature. The biodegradation process converts these complex synthetic dyes into simpler chemicals or sometime completely mineralized into elements. Most of the time secondary amines are generated in the biodegradation mixture; to monitor the degradation of effluents, HPLC analysis was performed before and after treatment. Available secondary amine including N-methylaniline, 3-methyldiphenylamine and phenylamine were used as standards of secondary amines. The retention time of N-methylaniline was t_R=3.57 min, that of 3-methyldiphenylamine was t_R=3.87 min, and that of diphenylamine was t_R=3.69 min (Fig. 4). Retention time of each compound depends upon their affinity towards column as well as the mobile phase used for elution (Iqbal and Asgher 2013, Sadhasivam et al. 2008). The formation of multiple peaks in treated samples at different time than in an untreated sample confirms the biodegradation of effluents (Lade et al. 2016).

Analysis of untreated MT industrial effluent through HPLC showed two well-developed peaks (t_R=3.475 min and t_R=6.09 min) and many small peaks. Treated MT effluent gave one peak of 3-methyldiphenyl-amine (t_R=3.87), two peaks of unknown compounds and many small peaks (Fig. 5). Untreated FST industrial effluent when passed through HPLC column

Table 3. Summary of biodegradation of textile industrial effluents after addition of different carbon and nitrogen sources

Biodegradation stage	Textile industrial effluents		
	MT effluent	FST effluent	ST effluent
Biodegradation (%) before C/N addition	83.02±1.43%	75.02±1.45 %	84±1.32%
Biodegradation (%) after C/N addition	93.21±1.73	86.81±1.81	95.03±1.63

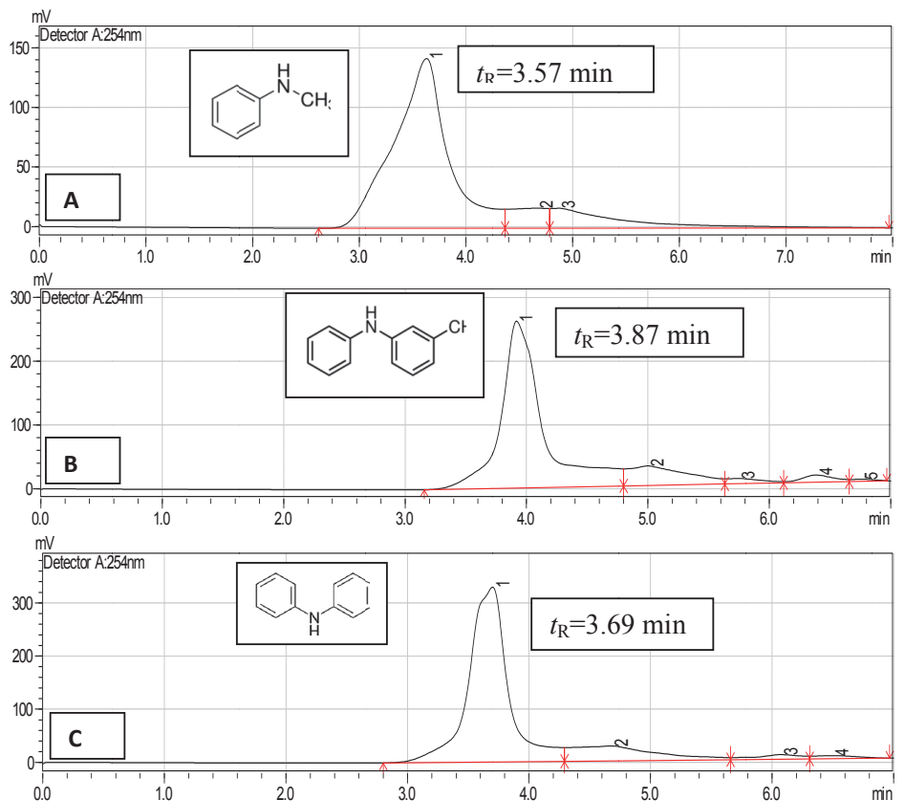


Fig. 4. HPLC chromatogram of standard secondary amines (A) N-methylaniline (t_R=3.57 min), (B) 3-methyldiphenylamine (t_R=3.87 min) and (C) diphenylamine (t_R=3.69 min)

gave three clear peaks and many small peaks, indicating the presence of a mixture of compounds in the effluent. Treated FST industrial sample degraded into diphenylamine (t_R=3.69), 3-methyldiphenylamine (t_R=3.87) and many other unknown compounds (Fig. 6). Analysis of ST industrial effluent with HPLC revealed that the untreated effluent gave three clear peaks (t_R=3.535 min, t_R=3.92 min and t_R=5.86 min) and 2 very small peaks. Treatment of effluents caused degradation of complex dyes into simpler compounds, results confirmed the formation of N-methylaniline and many other unknown compounds in treated effluents (Fig. 7). During our studies on biodegradation of disperse textile dyes, the production of 3-methyldiphenylamine and diphenylamine was observed in treated samples. This indicated that effluents under study mainly contain disperse dyes but exact composition is unknown. The basic aim of HPLC analysis was the confirmation of biodegradation into less-toxic or non-toxic byproducts which may make the treated effluent re-usable.

The secondary amines produced during the biodegradation of textile industrial effluents (Mujahid textile, Five star textile and Sitara textile) are less toxic. N-methylaniline may cause toxicity to organs if exposed for a very long period in high concentration and its carcinogenicity and mutagenecity have not been reported. Other amine 3-methyldiphenylamine may cause small irritation during long exposure but usually it is considered as non-carcinogenic and non-mutagenic. Diphenylamine, which generates in treated effluents, is non--carcinogenic and non-mutagenic but may cause little irritation or toxicity during long exposure to organs (Porwal et al. 2015, Saleh 2005, Wang et al. 2007).

Conclusions

It can be concluded that *C. puteana* IEBL-1, a brown rot fungus, is very efficient in biodegradation of textile industrial effluents under study. The addition of glucose, fructose,

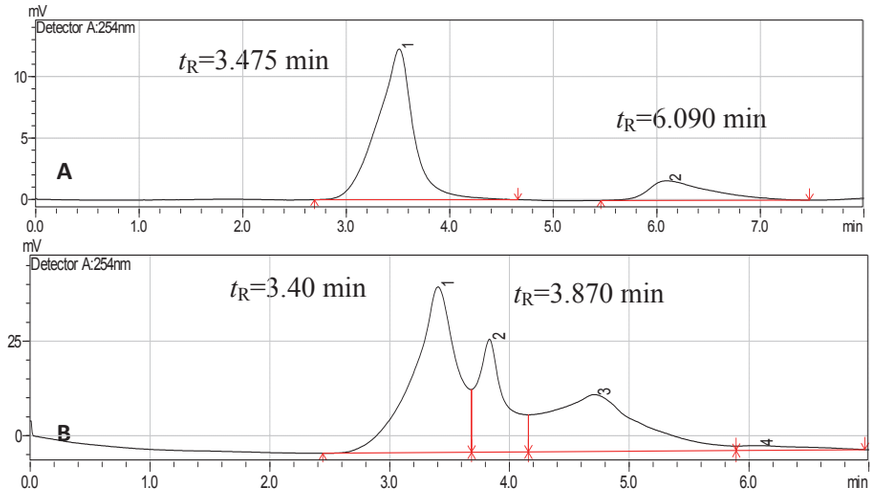


Fig. 5. HPLC chromatogram of MT industrial effluent (A) untreated sample, (B) treated sample, peak 2 for 3-methyldiphenylamine (t_R=3.870 min), peaks 1–3 for unknown compounds

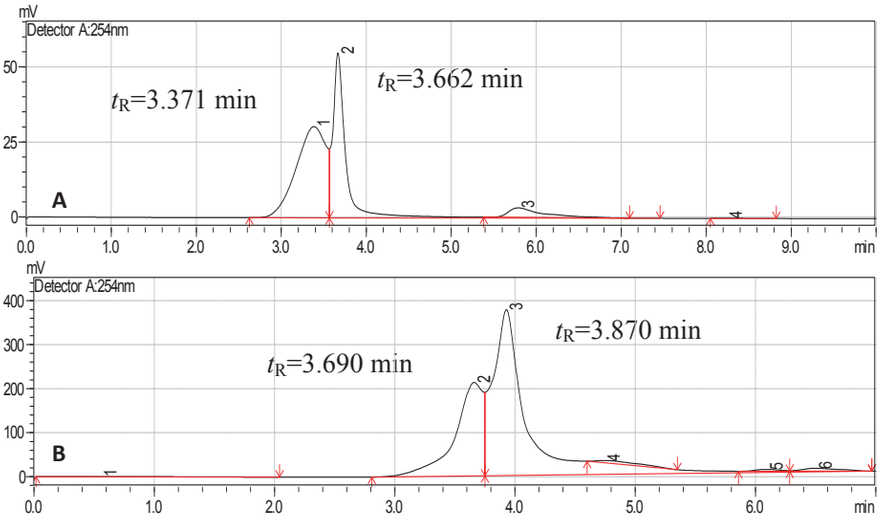


Fig. 6. HPLC chromatogram of FST industrial effluent (A) untreated sample, (B) treated sample, peak 2 for diphenylamine (t_R=3.690 min), peak 3 for 3-methyldiphenylamine (t_R=3.870 min) and peaks 4–6 for unknown compounds

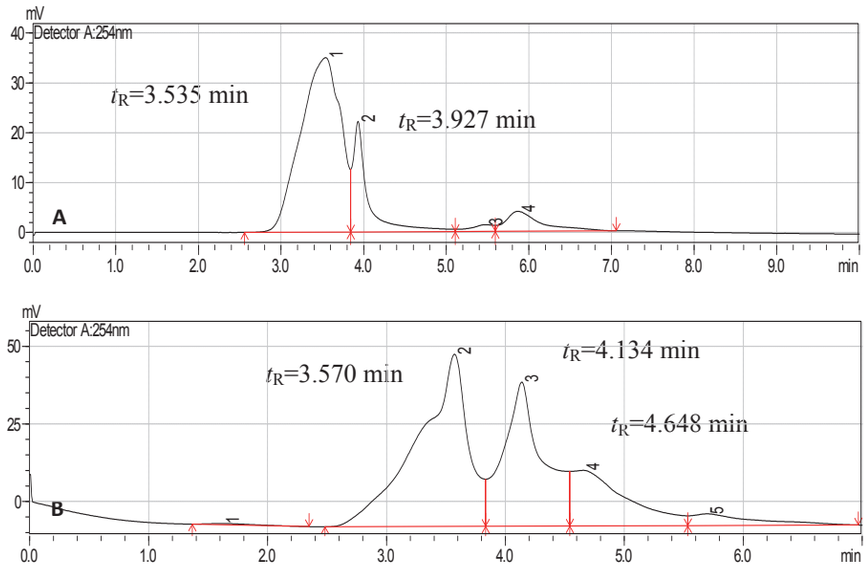


Fig. 7. HPLC chromatogram of ST industrial effluent (A) untreated sample, (B) treated sample, peak 2 for N-methylaniline (t_R=3.570 min), peaks 3–5 for unknown compound

ammonium sulfate, ammonium nitrate and ammonia as carbon and nitrogen sources, enhances the production of enzymes as well as the biodegradation of dyes in effluents. During the process *C. puteana* secreted the ligninolytic enzymes including laccase, lignin peroxidase and manganese peroxidase, which are actively involved in biodegradation process. There was a significant reduction, below recommended values, in BOD and COD observed after the treatment of effluents with the fungus. The production of less toxic secondary amine in small quantity, during the process showed that *C. puteana* IEBL-1 completely mineralized the dyes and indicated importance of this process. *C. puteana* IEBL-1 could be a very potential microorganism for wastewater treatment.

Novelty statement

First report on the use of *Coniophora puteana IEBL-1* for the biodegradation of textile effluents from AJK, Pakistan.

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