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Bioremediation of Reactive Blue 19 and Reactive Black 5 from Aqueous Solution by using Fungi *Aspergillus niger*

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A B S T R A C T

This study was aimed to investigate the potential of single culture of fungi *Aspergillus niger*, to decolorize reactive dyes from synthetic solution. Different parameters such as pH, time, temperature, agitation rate and various carbon, nitrogen and inorganic salts source, were optimized for decolorization of reactive blue 19 and reactive black 5 dyes. *Aspergillus niger* showed maximum dye decolorization under optimum condition and found to be more efficient when added in the dye solution of pH 8 and 10 with agitation at 130 rpm and incubation time for 7 days with 25°C. The results clearly showed that additional nutrient sources are effective in increasing dye decolorization rate. Fourier-transform infrared spectroscopy (FT-IR) investigated dyes before and after adsorption and data of the IR spectrum confirmed the presence of some functional groups in the dyes. The culture conditions were considerably optimized using Plackett-Burman statistical experimental designs. This study has confirmed that the potential *Aspergillus niger* in the decolorization of dyes and opened scope for the future analysis of their performance in the treatment of textile dyes.

Introduction

Synthetic dyes are being increasingly used in the textile, paper, cosmetics, leather dyeing, color photography, pharmaceutical and food industries because they can be easily produced and offer a larger variety of colors than natural dyes (Claus, 2002). The presence of even very low concentration of dyes in effluent is highly visible and degradation products of these textile dyes are often carcinogenic (Kim, 2003). Textile effluents are treated by physico-chemical methods that are often quite expensive. In addition, these methods do not generally degrade the pollutant, thereby causing an accumulation of

the dye as sludge creating a disposal problem. Over the past decade, biological decolorization has been investigated as method to transform, degrade or mineralize dyes. Moreover such decolorization is an eco-friendly method and cost comparative alternative to chemical degradation process (Verma and Madamwar, 2003). Currently, extensive research is focused to find optimal microbial biomass, which is as cheap as possible for the removal of contaminating dyes from large volumes of polluted water (Jadhav and Govindwar, 2006). For bioremediation of synthetic dye effluents,

several microorganisms, including bacteria and fungi can be employed. Fungi are recognized for their superior aptitudes to produce a large variety of extracellular proteins, organic acids and other metabolites and for their capacities to adapt to severe environmental constraints. *Aspergillus niger* showed varying decolorizing capacity to remove dyes from industrial effluents (Vidhiya and Padmalochana, 2015). Mathematical modelling and statistical analysis methods are versatile techniques for the investigation of multiple process variables because it makes the process easily optimized with fewer experimental trials (Claudia *et al.*, 2015). The Plackett Burman design (Plackett and Burman, 1946) method (PBD) is an effective screening design which considerably diminishes the number of experiments and Plackett- Burman design provides a fast and effective way to identify the important factors among a large number of variables, thereby, saving time and maintaining convincing information on each parameter (Abdel-Fattah *et al.*, 2005). Hence, the present study was aimed to evaluate the effects of culture conditions, represented as media components and environmental factors, on the decolorization of dyes by an isolate of *Aspergillus niger* fungi. This is needed to develop a near optimal medium in order to enhance the bioremediation process by means of statistically designed experiments is called Plackett-Burman experimental design.

Materials and Methods

Dyes and preparation of dye stock solution

Reactive dyes used in this study was purchased and used without further purification. The dye information was presented in table 1. A stock solution of 1000 mg/L was prepared by dissolving accurately weighed amounts of dyes in separate doses. The desirable experimental concentrations of solutions were prepared by diluting the stock solution with double distilled water.

Fungal isolate

The fungus used in this study was kindly provided by Microbiology lab, NIOF. It was purified and identified morphologically as *Aspergillus niger*. The spores *Aspergillus niger* were scrapped off from the agar plate surface and spore suspension was stored under refrigeration.

Fungal cultures

100 ml of basal media (1.25 g Glucose, 0.036 g urea, 2 g K_2HPO_4, 0.5g $MgSO_4$. $7H_2O$ in addition to 0.025 g of dyes per one liter. The medium was sterilized at 121°C for 15 minutes, under 15 Ib Pressure. Pure cultures of the provided fungi were grown in petri-dishes for 7 days using Minimal Salt Media (MSM). Fungal culture was tested for their ability to grow on MSM media with 1.5% agar and 1% of reactive dyes. Agar plates were incubated for 7 days at room temperature (28-30ºC) at a pH of 7.4±0.2.

Screening of the fungal strain for dye decolorization

The well-grown fungal colonies were screened for their dye decolorizing effect by inoculating them in 100 ml of the MSM containing of dye solution in 250 ml Erlenmeyer flask. At the end of the incubation period, culture was filtrated through whatman No.1 filter paper. These filtrates were measured by double beam UV-visible spectrophotometer to calculate decolorization percent. Decolorization activity was calculated according to the following equation (Moorthi *et al.,* 2007),

Decolorization (%) = $[A_O-A_I/A_O] \times 100$;
Where; A_o- initial absorbance; A_I - final absorbance.

Experimental designs (Plackett-Burman design) applied for optimization of dyes degradation by *A. niger*

The Plackett-Burman experimental design, a fractional factorial design, (Plackett and Burman, 1946) was used in this research to reflect the relative importance of various environmental factors on dyes decolorization in liquid cultures. In this experiment, ten independent variables (K_2HPO_4, $KHPO_4$, pH, Incubaton duration (hr), $MgSO_4$, temperature, spore suspension volume, urea, Na Cl, glucose) were screened in elven combinations organized according to the Plackett-Burman design matrix described in the results section.

The different factors were prepared in three levels: (-1) for the low level, (0) for medium level and (+1) for the high level.

Each row represents different experiment and each column represents different variables as shown in table 2.

The factors under investigation as well as levels of each factor used in the experimental design illustrated in table 3, all trials were performed in duplicates and the averages of decolorization observation results were treated as the responses. The main effect of each variable was determined with the following equation: $E_{xi} = (M_i^+ - M_i^-) / N$

Where: E_{xi} is the variable main effect, M_{i+} and M_{i-} are dyes decolorization percentages in trials where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum.

Results and Discussion

Effect of pH values on decolorization of reactive dyes

The results in figure 1A showed that the highest decolorization percentage of Reactive Black 5 at pH 10 was 84% and Reactive Blue 19 at pH 10 was 69 % with *A. niger*. In many studies, it was observed that the optimum pH for color removal is often at alkaline pH as Frida, (2009) mentioned that, the highest decolorization rates were obtained between pH 4 and 10. Willmott *et al*., (1998) reported that biological reduction of the azo bond can result in an increase in the pH due to the formation of aromatic amine metabolites, which are more basic than the original azo compound. The pH of an aqueous medium is a very important factor, it is affected by two criteria: firstly, since dyes are complex aromatic organic compounds having different functional groups and unsaturated bonds, they have different potential at different pH, resulting in the pH dependent net charge on the dye molecules. Secondary, the surface of fungi consist of many functional groups which are pH dependent (Hmd, 2011).

Effect of time on decolorization of reactive dyes

The effect of time course on decolorization of dyes under optimum conditions by *Aspergillus sp.* is illustrated in figure 1B. The decolorization percent has been increased by increasing the incubation period until reaching the optimum decolorization at the 7 day of incubation. Which, the maximum decolorization abilities of Reactive Black 5 and Reactive Blue 19 by *A. niger* with the following percentage: 93.8% and 79%, respectively.

Longer incubation periods revealed no a significant decolorization percentage at 10

days. This is may be due to depletion of nutrient from the medium and accumulation of some toxic secondary metabolite which inhibits fungal growth and show negative effect on overall dye degradation activity. Similar kind of result was obtained by Gopi *et al*., (2012).

Effect of temperatures on decolorization of reactive dyes

The results obtained are drowning stated in figure 2A. The decolorization percentage of Reactive Blue 19 and Reactive Black 5 was 84% and 91%, respectively at 25 °C with *A. niger*. Which, the optimum temperature was 25°C for maximum decolorization percentage. The optimum temperature for *Aspergillus niger* growth coincided with the maximum decolorization percentage. Higher temperatures caused a decrease in all those parameters, probably due to the production of large amount of metabolic heat thereby inhibiting microbial growth and enzyme formation (Iqbal and Saeed, 2007). The decreasing temperature may enhance the production of enzyme that increases the respiration rate and substrate metabolism. The degradation of pollutant by microorganisms relies on optimum temperature that favorably supports the microbial activity. Similar results were also shown by Haq *et al*., (2008) and Abedin, (2008) for the decolorization of crystal violet and malachite green by *Fusarium solani*.

Effect of agitation rate on dye degradation

The effect of agitation rate on dye degradation was determined and it was found that fungus *Aspergillus* shows increase in percent degradation activity with increasing rpm from 35 to 160 rpm as shown in figure 2B. Further increase in rpm shows negative effect on colour removal ability of fungus. Also, a higher color removal was observed in shaking cultures because of better oxygen transfer, where dissolved oxygen is considered to be an important factor which affects the decolorization process. The result is in agreement with the result obtained by (Gopi *et al.,* 2012) and Sheen, (2011) reported that an increase in the percentage uptake with increasing agitation rate due to reduction in film boundary layer of sorbent particles, which increased the external mass transfer coefficient, in addition mass transfer surrounding the sorbent particles, resulting in higher sorption rate.

Effect of nutrients sources on decolorization of reactive dyes by *A. niger*

Microorganisms require mineral nutrients such as nitrogen, phosphate and potassium (N, P and K) for cellular metabolism and therefore successful growth (Sihag *et al*., 2014). The addition of nutrients like carbon and nitrogen may increase the dye degradation efficiency (Shivannavar *et al.,* 2014).

Effect of carbon sources on decolorization of reactive dyes

The increase in dye decolorization after supplementation of carbon source is attributed to the fact that dyes are deficient in carbon content and biodegradation without any extra carbon and energy source is difficult (Padmavathy *et al*., 2003). Two carbon sources such as glucose and sucrose were used at 0.5 g/L. Figure 3 showed that glucose higher efficiency for decolorization percentage of Reactive Blue by *A. niger* with 75.3 % respectively, While sucrose showed higher efficiency in Reactive Black 5 dye, *with* percentage 79.5%. No dye decolorization was observed in the control flask without inoculum. Glucose plays multiple roles in dye decolorization mechanism which might be: the generation of H_2O_2 required for

extracellular peroxidase activity and/or the generation of Mn^{+3} complexing agents necessary for MnP activity (Kirk and Farrell, 1987).

Effect of different nitrogen sources on decolorization of reactive dyes

Nitrogen content had significant effect on fungal growth.The potential of *A. niger* fungi for decolorization of dye was checked by the addition of nitrogen sources (urea, ammonium chloride and ammonium molybdate) were used at 0.5g/L to estimate their effect on the decolorization efficiency of the fungal isolates. The addition of nitrogen sources was observed to have significant effect on degradation of three dyes as shown in figure 3. And the results clearly indicated that removal of dyes was greatly affected by addition of various nitrogen sources which found that highest decolorization shown by ammonium chloride and urea with percentage 49.33 and 48.52%, respectively. Mendez-Paz *et al.,* (2005) found that inorganic nitrogen supplement (NH_4Cl) suitable for decolorization of azo dye orange 7 under fed-batch and continuous anaerobic culture conditions.

Effect of inorganic salts source on decolorization of reactive dyes

Inorganic salts such as sodium choloride, potassium choloride and sodium nitroprusside were used at 0.5g/L to estimate their effect on the decolorization efficiency of the fungal isolates. Figure 3 showed that the highest decolorization of Reactive Black 5 and Reactive Blue 19 were 47.14 and 41.60 % occurred by sodium nitro prusside as inorganic salts. Dye manufacturing industries use large amount of salt in the manufacturing process, high salt concentration mainly causes osmotic imbalance thus, it is necessary to check the sustainability of the fungal isolate under high salt environment.

Fourier Transform Infrared Spectroscopy analysis (FTIR) of dyes wastewater

FTIR analysis was also performed for dyes effluents before and after fungal decolorization as described before, and FTIR analysis carried out for dyes wastewater presented in figure 4 A and B, which indicated that in the area of aromatic rings (800-400 cm^{-1}), one peak was at wavelength 430.35 cm^{-1} before bioremediation and disappeared after bioremediation which indicate wastewater colour removal (Hmd, 2011). The bands at 1055 cm^{-1} before bioremediation and 1080.20cm^{-1} after bioremediation were assigned to the –C–O stretching of alcoholic groups (Sheng *et al*., 2004). Peak at 1055.17cm^{-1} before bioremediation was shifted and increased in intensity to 1080.20 cm^{-1} this may be due to M-O stretching Alumina, K, Ca, Mg. Also, one peak at 1443.59 cm^{-1} was disappeared which indicated that further degradation were occurred after bioremediation. The other peaks were C=O stretching carboxylic acids at 1635.39 and 1636.68 cm^{-1}. Peaks in untreated dyes was seen at 2080.46 cm^{-1}, this is due to N-H and C=C str. Frequency (Pratheebaa *et al.,* 2013) but slight changes were observed in corresponding peaks after treatment and was seen at 2074.20 cm^{-1}, hence these corresponding groups might be involved in the decolorization process. From the FTIR analysis it was found that O-H stretching (Intermolecular hydrogen bonded OH) peak shifted from 3446.13 cm^{-1} to 3451.23 cm^{-1} for adsorbed fungi. The FTIR spectroscopic analysis indicated broad bands at 3370–3410 cm^{-1}, representing bonded –OH stretch representing the presence of hydroxyl bond and –NH groups this may be due to formation primary and secondary amines (Sheng *et al*., 2004). From FTIR study, the formation of new absorption bands, the change in absorption intensity, and the shift in wavenumber of functional groups could be

due to interaction of ions of dyes with active sites of biosorbents. Results from this study suggest, carbonyl, hydroxyl and amine are the main adsorption sites in *A. niger.*

Table.1 Some chemical properties about the studied dyestuffs

Commercial name (colour index name)	Chemical structure and molecular weight	λ max
Remazol Black GF (Reactive Black 5)	**Molecular weight: 992**	**597-600**
Royal Blue E-FR (Reactive Blue 19)	Molecular weight**: 566**	**620**

(* Reference; Abadulla et al., 2000).

Table.2 List of different variables under study and their coded levels

No	Factor	Factor Name (stock-g/l)	Conc %	Low	Conc %	Medium	Conc %	High
1	A	glucose - 5%	0.5(**w/v**)	-1	2	0	4	+1
2	B	MgSO4.7H₂O 1%	0.1(**w/v**)	-1	0.5(**w/v**)	0	0.9	+1
3	C	Urea-1%	0.1(**w/v**)	-1	0.5	0	0.9	+1
4	D	(KHPO₄)- 1%	0.1 (**w/v**)	-1	0.5	0	0.9	+1
5	E	(K₂HPO₄)- 1%	0.1 (**w/v**)	-1	0.5	0	0.9	+1
6	F	NaCl - 1%	0.1 (**w/v**)	-1	0.4	0	0.8	+1
7	G	Spore suspension volume	0.2 %	-1	0.3 %	0	0.5 %	+1
8	H	pH (alkaline- acidic)	4	-1	7	0	8	+1
9	I	Incubaton duration (hr)	168	-1	240	0	336	+1
10	J	Temperature	20 °C	-1	Room(25°C)	0	35 °C	+1

Table.3 Plackett-Burman design for medium optimization, statistical analyses and measured response Variables (factors)

no.	Mean	influencing factors										Response
		1	**2**	**3**	**4**	**5**	**6**	**7**	8	**9**	**10**	RSD%
		A	B	C	D	E	F	G	H	I	J	
1	+	+	-	+	-	-	-	+	+	+	-	98.28
2	+	+	+	-	+	-	-	-	+	+	+	66.20
3	+	-	+	+	-	+	-	-	-	+	+	14.52
4	+	+	-	+	+	-	+	-	-	-	+	32.33
5	+	+	+	-	+	+	-	+	-	-	-	82.33
6	+	+	+	+	-	+	+	-	+	-	-	77.96
7	+	-	+	+	+	-	+	+	-	+	-	19.07
8	+	-	-	+	+	+	-	+	+	-	+	38.77
9	+	-	-	-	+	+	+	-	+	+	-	53.30
10	+	+	-	-	-	+	+	+	-	+	+	46.33
11	+	-	+	-	-	-	+	+	+	-	+	8.20
12	+	-	-	-	-	-	-	-	-	-	-	81.90
Main effect (%)		15.64	-6.88	-2.74	-2.24	1.28	-12.75	-3.45	4.83	-1.29	-17.89	
t-state		1.28	-5.42	0.87	0.032	1.103	0.86	0.71	-1.98	1.0	-2.45	
t-critical		1.43	1.34	1.39	1.47	1.47	1.41	1.41	1.41	1.41	1.43	

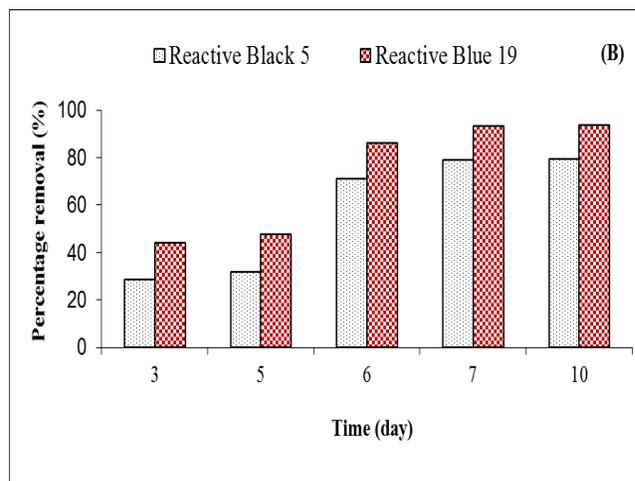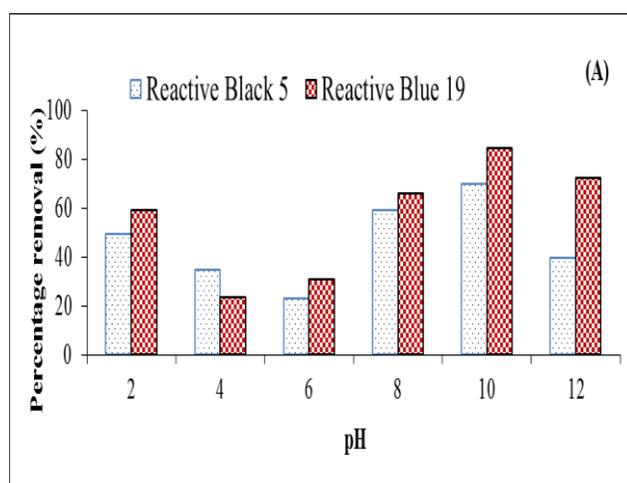**Fig.1** Effect of pH (a) and time (b) on decolorization of dyes by *A. niger*

Fig.2 Effect of temperature(a) and agitation rate (b) on decolorization of dyes
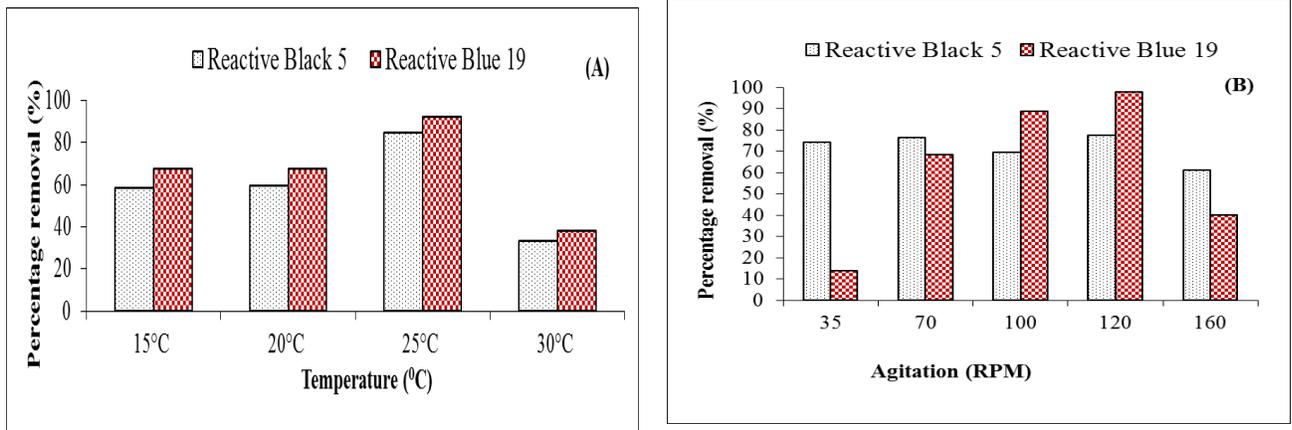

Fig.3 Effect of different nutrients sources on decolorization of reactive dyes
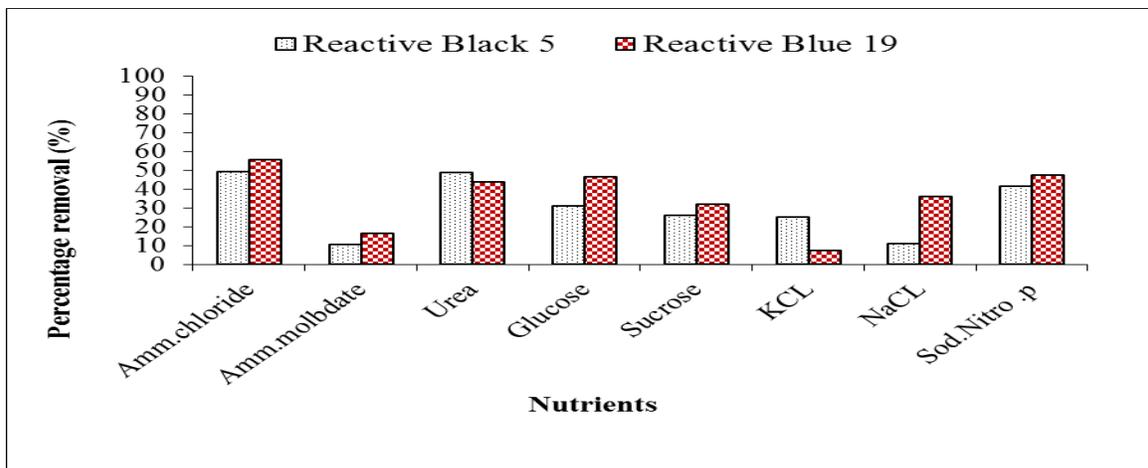

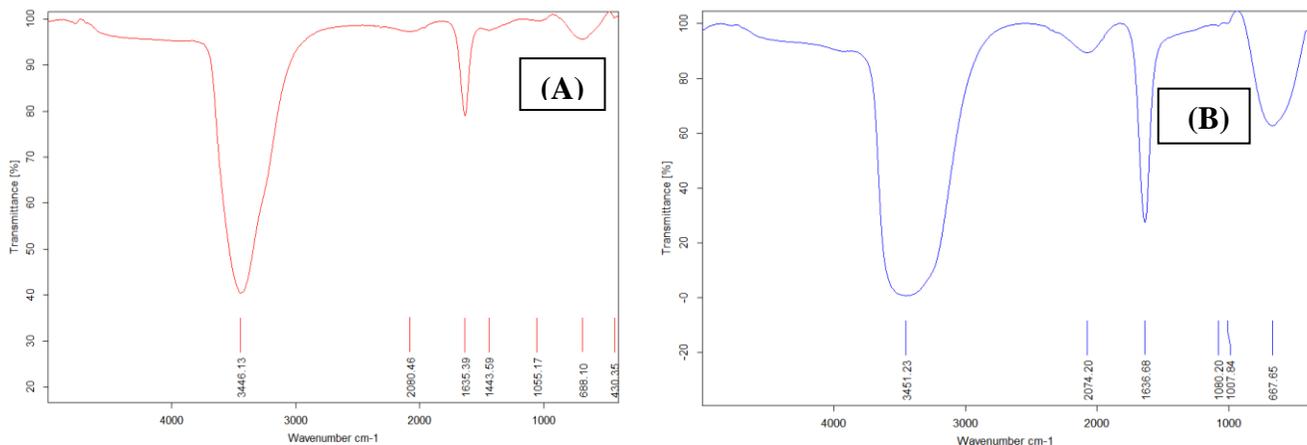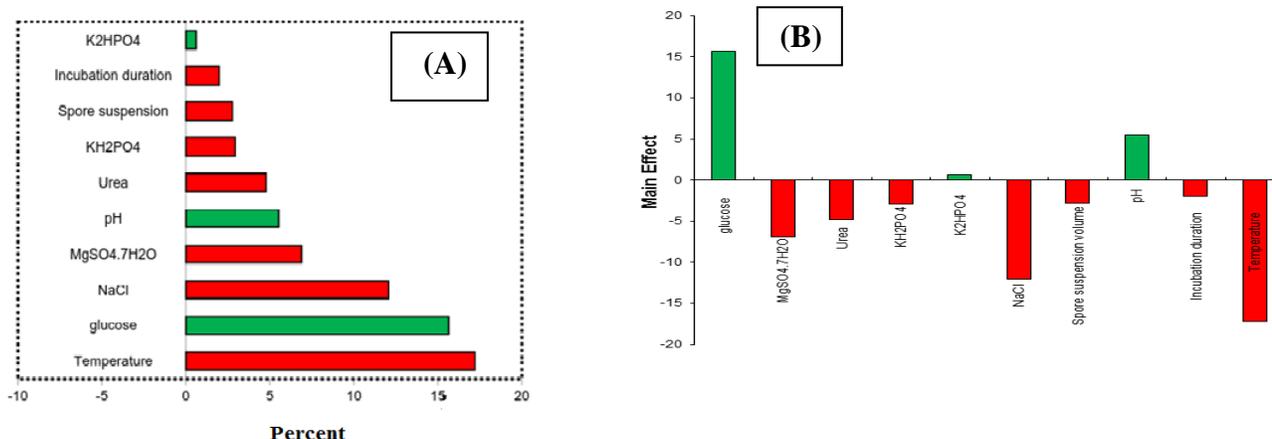**Fig.4** FTIR spectrum of *A. niger* before (a) and after (b) bioremediation

Fig.5 Pareto plot for PB parameter estimates (a) and effect of independent variables on mixed reactive dyes removal by *A. niger* based on Plackett-Burman design results

Screening of important variables using Plackett-Burman experimental design

Plackett–Burman designs are experimental designs presented in 1946 by Plackett and Burman while working in the British Ministry of Supply (Plackett and Burman *et al.*, 1946). Their goal was to find experimental designs for investigating the dependence of some measured quantity on a number of independent variables (factors), each taking L; levels, in such a way as to minimize the variance of the estimates of these dependencies using a limited number of experiments. Interactions between the factors were considered negligible. The solution to this problem is to find an experimental design where each combination of levels for any pair of factors appears the same number of times, throughout all the experimental runs as table 2. A complete factorial design would satisfy this criterion, but the idea was to find smaller designs.

This model describes no interaction among factors and issued to screen and evaluates the important factors that influence dyes bioremediation and fungal growth. In this study, a 12-run Plackett-Burman design was applied to evaluate ten factors; K_2HPO_4, $KHPO_4$, pH, Incubation duration (hr), $MgSO_4$, temperature, spore suspension volume, urea, NaCl, glucose

and dyes were selected for the screening process by PB design. The data listed in table 2 indicated a wide variation in dyes degradation from 8.20 % to 98.28 %, in the 12 trials.

Figure 6A and B shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the significant variables and the magnitude of each factor estimate (independent on its contribution, either positive or negative) and is a convenient way to view the results of a Plackett-Burman design. The highest positive significant variable for decolorization of dyes is glucose when compared to other factor. While, temperature and NaCl have showed the maximum negative effect in the growth medium, the supplementation of glucose has two reasons; first it promotes the growth and rapid establishment of the fungus. Second, in the presence of lignin, the fungus utilizes carbon sources more easily.

Analysis of variance (ANOVA) was performed on the data to determine the significance of fitted model and to test the significance of the effect of individual parameters on dyes removal. Statistical analysis of the regression coefficients and the *t*-values of 10 factors of the data (*t*-test) showed that the variable with confidence level about 90% is considered as significant parameter. It was clear that variables

temperature, pH and MgSO$_4$, were the significant factors, while, variables; KH$_2$PO$_4$, glucose, spore suspension volume, K$_2$HPO$_4$, induration time, urea and NaCl with confidence levels about 90%, were considered insignificant as shown in table 3. The temperature required to produce the maximum rate of color removal tends to correspond with the optimum cell culture growth temperature (Pearce *et al.,* 2003), and its statistical significance was checked by Fischer's F-test. The statistical analysis of the Plackett-Burman design demonstrated that the model the "Model F-value" of 6.83 implies the model is not significant relative to the noise.

The fitness of the model was examined by the coefficient of determination R^2, which was found to be 0.841, which although is not that high as the coefficient of determination of the residual dyes response determined by weight, but it is good enough to explain the variabilities of the data. The model was found to be adequate for prediction within the range of variables employed. The coefficient of variation (CV) indicates the degree of accuracy with which the treatments are compared. The lower value of CV (23.079 %) demonstrated that the performed experiment was highly reliable.The created model could be used to predict the response dyes removal percentage when using different culture conditions.

In conclusion, *Aspergillus niger* was able to perform reactive blue 19 and reactive black 5 dyes decolorization under wide range of conditions, viz., pH (2–12), incubation time (3-10 days), temperature (15–30 °C), agitation rate (35-160) and some nutrients sources had a major influence on dye removal by *A. niger*. FT-IR analysis indicated the presence of carbonyl, hydroxyl and amine as functional groups and the main adsorption sites for reactive dyes solution. This study investigated the effect of some parameters on the biodegradation efficiency of reactive dyes by a selected strain of *A. niger* by using a statistical analysis of design experiments methodology (Plackett- Burman) to get the maximum results with a minimum of experiments and screening the factors with significant influence. Thus any bioprocesses based dye removal system using such type of fungus should be design on the basis of these parameters for successful operation.

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