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Original article

Development of efficient strain of *Ganoderma lucidum* for biological stripping of cotton fabric dyed Reactive Blue 21

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ABSTRACT

One of the most common dyeing problems of textile industries is uneven and faulty dyeing over the finished quality of fabrics due to different reasons. These problems are usually tackled through chemical degradation in which uneven and faulty dye is removed from the surface of fiber but fabric quality is compromised. Chemical process also reduces the strength of the fabric and durability of textile material by reduction in reactive dye ability. The fabric cannot be reused due to the reduced strength. To overcome above mentioned problem, biological method of stripping in which enzymes produced by different micro-organisms are used. This process has no harmful effect on the fabric and is safe for environment. In this research work reactive blue 21 dye with 0.5, 2 and 4% shade strengths was used to dye cotton fabric. The *Ganoderma lucidum* fungal strains were mutated by UV mutagen, and five were selected for further processing. These mutant strains were grown at temperature ranges (20 °C to 40 °C); pH(3–5); inoculum size(1–5 mL) and fermentation time (3–15 days) . The required nutrients media to produce the ligninolytic enzymes was added to the flask. The strain which gave the fast decolourization results was selected for further optimization. Optimization was done by observing the variables: incubation time 12 days, pH 4, temperature 30 °C, and inoculum size 3 mL by applying Response Surface Methodology (RSM) in Central Composite Design (CCD). During the process of fabric color stripping, the enzyme assay revealed that the respective mutant UV-60 strain produced active enzymes with their V_{max}, Mnp (427U/mL), LiP (785U/mL), and Lac (75 U/mL) enzymes decolorized 89% of the dye which is 25% more than the parent strain and also the production of enzyme is Mnp (344U/mL), LiP (693U/mL), and Lac (59 U/mL) enzymes which is lower than mutant strain.
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1. Introduction

Different types of dyes and their mixtures are used to dye various clothes to develop attractive colours and shades (Campos et al., 2001). Dyeing of clothes is the most important and common one before food colouring. Clothes are the basic need of humankind, and dyes add different color shades and beauty to them. Natural dyes has been used for the dyeing of clothes at a small scale in the last few decades, but after the development of synthetic dyes, the process was adopted on a large scale in textile industries. Dyes are also used in leather, food, paper, and plastic (D'Souza et al., 2006).

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Due to wide range of colour shades, ease of application, low consumption of energy and good colour strength, reactive dyes are mostly used in textile industries. Reactive dyes have chromophoric group that combine with different reactive groups like vinyl sulfone, chlorotriazine, trichloropyrimidine and difluorochloropyrimidine (Asgher et al., 2010). Due to poor fixation these dyes when discharged in to the water causes major environmental problems because dyes contain many acidic, basic and aromatic groups that produce toxic, carcinogenic and mutagenic substance that are difficult to degrade. These dyes also hinder the penetration of light through water and thus adversely affect the aquatic plants and photosynthesis

With the advancement in dying techniques, it is necessary to resolve the dye's errors or unrequired stains during the dyeing process. For many years a lot of chemical and physicals methods remained in practice to remove the uneven dyes. Nevertheless, all these techniques harm the strength of the fibre of fabric as well as they have high operational cost and cause environmental pollution (Shinkafi et al., 2015). However, the advent of biological methods brought a revolution in this sector. Many new biological tools have replaced the traditional and harmful methods to help humankind in every aspect of life due to their reliability, economics, and efficiency. For this purpose, microbes are of prime importance. The bacteria and fungi produce such biologically active compounds that can decolorize and metabolite the dyes as a biological and environmental friendly color stripping technology (Chatha et al., 2016).

Among these microorganisms, WRF are more commonly used for degradation of dye (Khehra et al., 2005). White Rot Fungi (WRF) are considered to be best dye-degraders than others because of their extracellular ligninolytic enzyme system capable of degrading dye and other organic pollutants. Large quantities of extracellular ligninolytic enzymes produced by the fungi help to remove dyes from industrial effluent and from the dyed fabrics in case of uneven dyeing for recycling of the clothes (D'Souza et al., 2006). Different species of yeast are used as a decolorizing agent for various textile dyes, including azo dyes. To bleach out the azo dyes, application of *Candida tropicalis* was found to be succesful for complete decolorization of Reactive Black 5 dye with 200 mg/L in less than 24 h (Jafari et al., 2012). The amount of dye decolorized varies with the change in conditions. In an earlier study, Meehan et al. (2000) found that Remazol Black B dye can be efficiently decolorized by *Kluyveromyces marxianus* with the maximum rate of decolorization beeing achieved at 37 °C. *Aeromonas hydrophilla* can also be employed for the removal of azo dyes (Chen et al., 2003). Bioflocculant producing bacterias also decolorize the dye with 80 to 97% decolorizing efficiency. The use of bacteria is safe for the environment, produce less waste or residue, which has replaced the other physical and chemical methods, but when the azo bond is cleaved in the chemical structure of dye, bacterial azo reductase causes the production of carcinogenic amines that makes its use limited (Arutchelvan et al., 2004; Khataee and Kasiri, 2010).

Many studies have been carried out on fungi for the use of decolorization of dyes after yeast and bacteria. *Aspergillus niger* was used to decolourize the dyes due to biosorption (Çetin and Donmez, 2006). Fungi cause biosorption of the chemical structure of the dye. Fungi produce laccase enzymes, manganese peroxidase and lignin peroxidase to efficiently decolorize azo dyes, heterocyclic dyes, aromatic structure in dyes and any other chemical that is present without producing any toxic compound (Yan et al.,2014). Fungi can easily decolorize Acid dyes used for dyeing wool and silk and Reactive dyes used for dyeing cotton at optimal pH 4–5. Most commonly, white-rot fungi have an extracellular enzyme system that can bleach out the dyes (Camarero et al., 2005), and it is eco-friendly and cost-effective technology (Campos et al., 2001).

White rot fungi (WRF) belong to *Basidiomycetes* and can decolorize the toxic organic compounds using extracellular ligninolytic enzymes (Asgher et al., 2013). White rot fungi are eukaryotic microorganisms that are excellent dye-degraders because of their extracellular ligninolytic enzyme system, making them efficient degraders of dye and other organic pollutants. They can break dye due to a lack of substrate specificity (Wesenberg et al., 2003). WRF produces large quantities of extracellular ligninolytic enzymes, separate dyes from the industrial outflow and dyed fabrics regarding potholed dyeing to reproduce the garments (Kaushik and Malik, 2009). WRF are a potent bioremediation agent due to their high tolerance rate for a toxic environment. Strains of WRF grow on low-cost and straightforward media. WRF is divided into the following three groups based on the extracellular enzyme system they secrete during dye degradation (Khehra et al.,2005). These are (i) LiP- MnP and laccase-generating, (ii) MnP and laccase manufacturing and (iii) LiP and laccase producing fungi (Ghasemi et al., 2010).

Ganoderma lucidum, a WRF, can decolorize synthetic dyes (Ghasemzadeh et al., 2011). It is selected due to its easy growth on solid media that leads to degradation. Due to its growth in the low-cost medium, it is mutated for the hyperproduction of enzymes. It can decompose various aromatic compounds under optimum physical and nutritional conditions (Batool et al.,2013). *Ganoderma lucidum* was reported to be efficient for decolorization of C.I. reactive black 5 with up to 70.81% color stripping of dyed fabric (Chatha et al., 2014).

Laccase is a copper containing enzymes that oxidized several aromatic compounds especially phenols and anilins by the reduction of molecular oxygen in water. It is naturally present in the plants, fungi and insects. Laccase are considered to be a potential solution for textile effluent problem because of its ability to degrade dyes. Laccase are also used in stone-washing along with cellulose to hamper fiber damage. It is also used in microfuels and in the development of biosensors. It also act as bioelectrocatalyst. Yang et al. (2014) examined a white rot fungus have very high laccase level. Laccase belongs to the oxidases class of enzyme. This white rot fungus grows in the shaking flask for several days. Laccase became stable at pH 5 in the presence of organic solvent several metal ions Na^{2+}, K^{+}, Ca^{+2}, Mg^{+2} and Zn^{+2} enhanced its activity. Laccase play important role in degradation of dye and textile waste. Due to high yield and ability of decolorization of dye laccase is very useful for industries and environment protection

Ganoderma lucidum fungal strain was used in the present study. This study's objectives are:to produce an effective mutant strain of *Ganoderma lucidum* to decolorize diverse concentrations of Reactive Blue 21; to evaluate mutant strains' capability to decolourize dye through their enzyme system and optimize the procedure of colour stripping using selected fungal mutant. Removing the color from an uneven or faulty dyed fabric without damaging it in economical ways is the main focus of this study

2. Material and methods

2.1. Microorganism

The fungus *Ganoderma lucidum* available in the Industrial Biotechnological lab (IBL), Biochemistry Department, University of Agriculture, Faisalabad, was used for biological stripping of dyed cotton fabrics.

2.2. Slants preparations

The PDA medium was prepared in a 250 mL flask, and pH was kept at 4.5 using M HCL/ M NaOH, and the pH was monitored with

a pH meter (Inolab WTW Series, A070601185; Germany). The PDA solution was placed into the uncontaminated test tubes plugged with cotton, wrapped with the aluminium foil and autoclaved for sterilization (Sanyo MLS-3020U, Japan) at 119 ± 2 °C for 15 min. Finally, for the solidification of slants, the test tubes were positioned slanting for 24 h at ambient temperature (Fig. 2).

2.3. Multiplication of fungus

The pure culture of *G. lucidum* was transferred aseptically into the test tubes with the sterilized inoculation loop in the clean laminar airflow (Dalton; Japan). For the inoculated slants' growth, the test tubes were incubated for five days at room temperature. The slants with the growth of fungus were refrigerated (4 °C). Cultures were refreshed periodically during research.

2.4. Preparation of inoculum

Inoculum of *G. lucidum* was made via inoculating the fungus in sterilized Kirk's basal medium at pH 4.5. After cooling. *G, lucidum* spores from slants were transferred to the inoculums medium in laminar airflow. For 5–7 days, the flask was kept under incubation (30 °C) in an orbital shaker (120 rpm) to get a homogenous spore suspension containing 10^7-10^8 spores/mL.

2.5. Mutagenesis for hyper producing strain development

2.5.1. UV mutagenesis

A UV germicidal lamp of 20 W (Philips) was used for UV irradiation mutagenesis of *G. lucidum*. The aseptic suspension of spores was placed into flat bottom sterile Petri plate. The plates were sterilized by UV rays kept at 10 cm for 30, 60, 90, 120 and 150 min. The Petri plates were uncovered, occasionally mixed and kept in the dark for overnight to avoid photoreactivation (Fig. 3).

2.5.2. Mutant selection using deoxy-D-glucose

After treating the spores with UV light, the spore dilution (0.1MI) was expanded on PDA media containing deoxy-D-glucose in a dark room. Few mutants appearing on each plate were picked with a sterilized toothpick and transferred to new PDA Petri plates containing Triton X-100.

2.5.3. Plate screening method

The spore dilution (0.1 mL) was placed on PDA media consisting of 2% Triton X-100 as colony restrictor in the darkroom. Spores without treatment were also kept as control. Strict aseptic conditions were followed for carrying out the whole process in laminar airflow. Lids were tightly placed over the plates and incubated at 37 °C for 3–7 days. The selected colonies from each plate were used for the biological stripping of dyed cotton fabric.

2.6. Collection of materials and dying of fabric

RB21 (Reactive Blue 21) dye having a powdered blue appearance with a molecular weight of 1390 g/mol was donated by Sandal Dyestuffs (Pvt.) Limited, Pakistan (Faisalabad) (Fig. 1). The knitted cotton textile was procured from the hosiery market, Jinnah Colony, Pakistan (Faisalabad).

2.7. Cotton fabric dyeing

Grey fabric was bleached before dying by 50% H_2O_2 solution, which was diluted to 20 g/L and a solution of 50% NaOH, which was diluted to 15 g/L at 50 °C for for 30 min. The fabric was then dyed with RB21 in different shade strength (0.5, 2 and 4%) as per the weight of fabric (4 g), followed by the traditional exhaust dyeing protocol. The ratio of fabric to dying solution was kept at 1:15 for requisite shade strength. For a 0.5% shade strength, 2 mL dye solution was made, and 1.2 g of Na_2SO_4 and Na_2CO_3 were added. For 2% shade strength, 8 mL dye solution was made, and 1.5 g each of Na_2SO_4 and Na_2CO_3 were added, and for 4% shade strength, 16 mL dye solution was added with 1.8 g each of Na_2SO_4 and sodium Na_2CO_3. Dyeing was done at 45 °C for 15 min, and then the temperature was increased to 60 °C for one hour. After dyeing, the fabric was washed thrice with water and dried afterwards

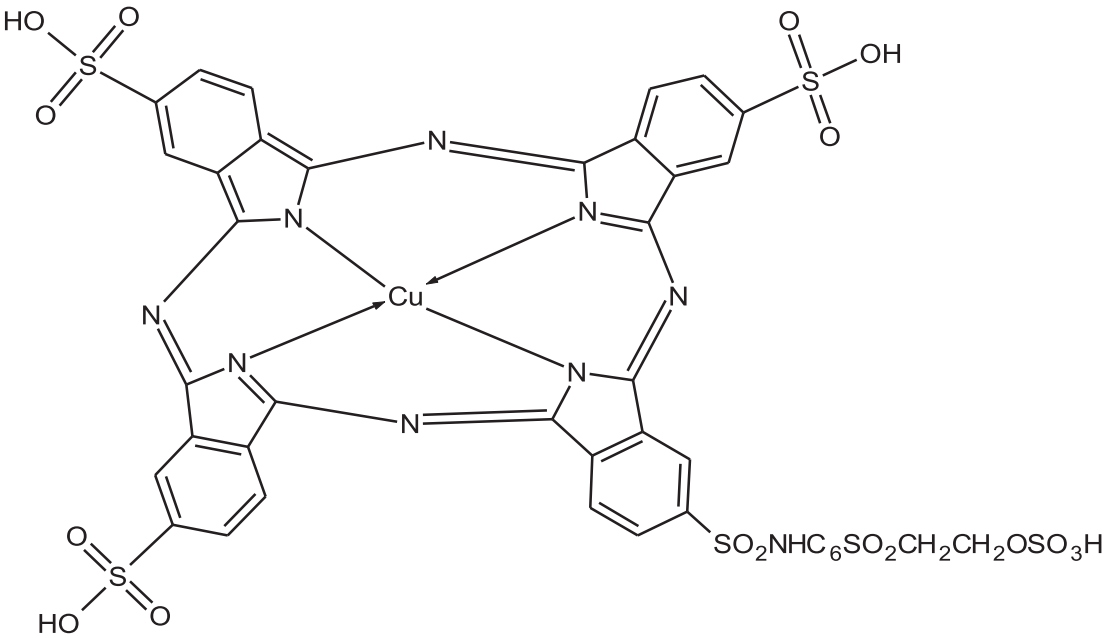


Fig. 1. Structure of Reactive Blue 21.

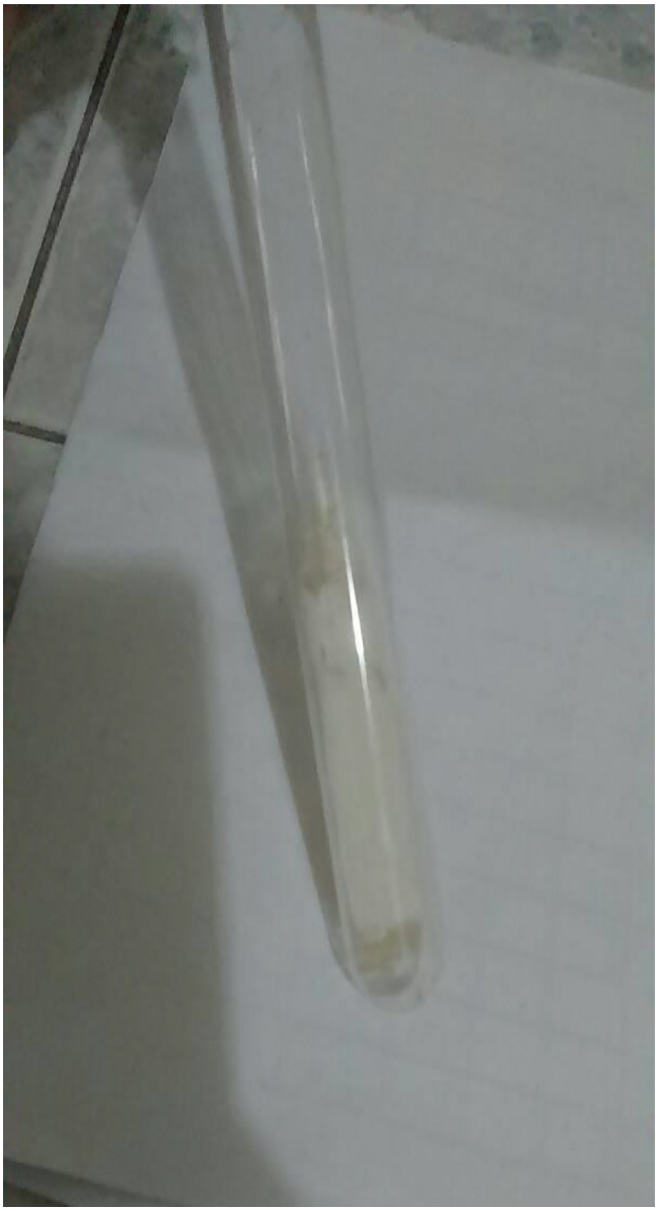


Fig. 2. Growth of Ganoderma lucidum on slant.

2.8. Biological color stripping

2.8.1. Preparation of inoculum from mutants

Homogenous spore suspension of selected *G. lucidum* mutants was made through inoculating the fungus in Kirk's basal medium.

2.8.2. Biological color stripping procedure

The three different shade strengths of dyed fabric were subjected to color stripping under favourable conditions. Triple flask for each shade strength with One hundred millilitres of Basel medium was prepared following the method of Chatha et al. (2014) 1 g of wheat bran ,peptone, $MnSO_4$ and $ZnSO_4$ were prepared. The 3×3 in. dyed fabric fragments of three shades (0.5, 2 and 4%) were added to flask, and pH was adjusted to 4.0 with M NaOH/M HCl. The prepared experimental medium was autoclaved. Two-millilitre inoculums in triplicate were inoculated in three stripping flasks with each mutant of *G. lucidum* triplicate flask were prepared for parent strain. The prepared inoculum was incubated for 15 days at 35 °C in a shaking incubator at 120 rpm. After 15 days of incubation, the triplicate samples and control flasks were taken out, and fabric pieces were thoroughly washed, dried, labelled and examined for color stripping (Fig. 4).

2.8.3. Measurement of colour stripping

With the spectra flash spectrophotometer at Government College University, Faisalabad, the strength of colour of both stripped and the unstripped cotton fabric was observed. K/S value was used to determine the colour strength of the dyed fabric K/S value is the ratio of light absorbed and reflected from the fabric. The formula calculated percentage decolorization:

$$\% \text{ stripping} = \text{K/S value of controlled sample} - \text{K/S value of stripping sample/K/S value of unstripped fabric} \times 100$$

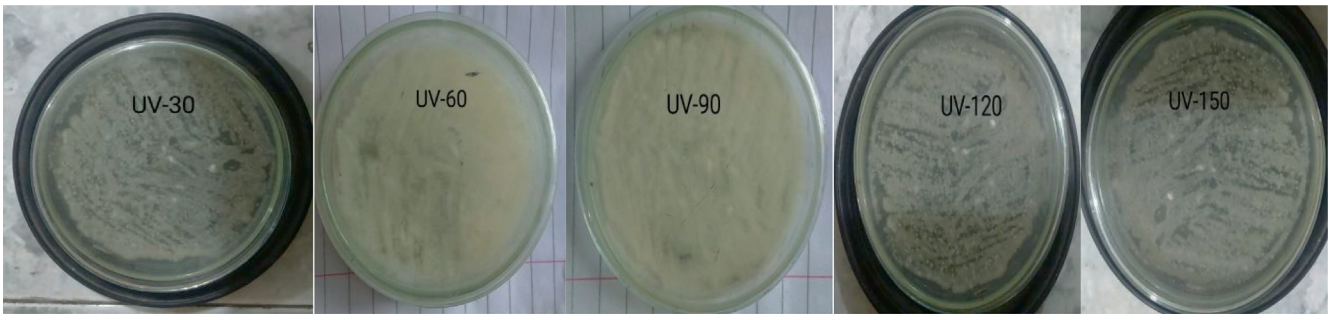
The quality of the stripped and the unstripped value was determined by using the below-mentioned tests:

2.8.4. Bursting strength

This test determined the resistance of the fabric to bursting by using a pneumatic diaphragm bursting tester. This test applies to broad collections in the textile industries and measured in the SI unit or inch-pound unit. The strength of bursting of biologically stripped and unstripped fabric was measured at National Textile University, Faisalabad, following the American Association of Textile Chemist and Colorist.

2.8.5. Pilling

Pilling is the small ball-like structure on the surface of the fabric due to broken fiber. The Pilling of biologically stripped and unstripped method was determined by Martindale tester at



(A) (B) (C) (D) (E)

Fig. 3. Growth pattern of UV irradiated mutants of Ganoderma lucidum on petri plates (A) GL-30 (B) GL-60 (C) GL-90 (D) GL-120 (E) GL-150.

Fig. 4. Prepared flasks with all the nutrient medium and dyed fabric.

National Textile University, Faisalabad. It followed the technique of American society testing material.

2.9. Enzyme profile

Liquid biomass from each flask after ten days of incubation was collected and filtered. The filtrate was centrifuged for 20 min at 120 rpm to separate the debris. Supernatant from each flask was analysed for laccase, Manganese peroxidase and lignin peroxidase (LiP) to determine the important enzyme behind decolorization.

2.10. Enzyme activity assay

2.10.1. Lignin peroxidase (LiP)

The method of Tian et al. (2013), was used to check the activity of Lignin peroxidase (LiP) following the H_2O_2 dependant oxidation of veratryl alcohol to veratraldehyde at 25 °C and 310 nm wavelength.

2.10.2. Manganese peroxidase (MnP)

For Mnp assay, the procedure of Sumandono et al. (2015) was followed. The assay substrate $MnSO_4$ was added to sodium malonate buffer (pH 3) in the presence of H_2O_2. Manganese ions Mn^{+3} form a complex with malonate that absorbs at 270 nm. (C_{270} = 11590 M^{-1} cm^{-1}).

2.10.3. Laccase (Lac)

Laccase was assayed by monitoring the oxidation of 2,2 azinobis (3-ethylbenzthiazoline)- 6 sulphonate (ABTS) (Tian et al., 2013).

2.11. Optimization of different factors for maximum decolorization by Response surface Methodology (RSM)

After decolorization of fabric by different mutants, the mutant showing best colour removal was selected. Various fermentation process parameters affecting fungal growth and enzymes production during dye colour removal were optimized. The parameters optimized were (1) Incubation temperature (25 to 35 °C); (2) pH (3 to 5); (3) fermentation time (120–150 h) and (4) Inoculum size (3-6Ml) (Table 1).

3. Results

3.1. UV mutagenesis

In support of UV mutagenesis, the fungus's homogenous spore suspension was made in a 250 mL Erlenmeyer flask. UV irradiation mutagenesis was used on *Ganoderma lucidum* spores for different time intervals varying from 30 to 150 min (Fig. 4.1). Semi dark-room was used for UV irradiation. By increasing the irradiation

Table 1
Central Composite Design matrix of independent variables with Response Surface Methodology.

Runs	A: pH	B: Temperature (°C)	C: Inoculum size (mL)	D:Fermentation time (Days)
1	3.00	20.00	5.00	15.00
2	4.00	30.00	3.00	9.00
3	3.00	20.00	1.00	15.00
4	4.00	30.00	3.00	9.00
5	4.00	30.00	3.00	6.00
6	3.00	40.00	1.00	3.00
7	4.00	30.00	3.00	9.00
8	3.00	40.00	1.00	15.00
9	5.00	20.00	5.00	15.00
10	3.00	40.00	5.00	15.00
11	4.00	30.00	3.00	9.00
12	5.00	40.00	5.00	15.00
13	3.00	20.00	1.00	3.00
14	4.00	35.00	3.00	9.00
15	5.00	20.00	1.00	15.00
16	4.00	30.00	4.00	9.00
17	5.00	20.00	1.00	3.00
18	3.50	30.00	3.00	9.00
19	3.00	20.00	5.00	3.00
20	4.00	25.00	3.00	9.00
21	4.00	30.00	3.00	9.00
22	5.00	40.00	1.00	15.00
23	5.00	40.00	5.00	3.00
24	4.00	30.00	3.00	9.00
25	3.00	40.00	5.00	3.00
26	4.50	30.00	3.00	9.00
27	4.00	30.00	2.00	9.00
28	5.00	40.00	1.00	3.00
29	5.00	20.00	5.00	3.00
30	4.00	30.00	3.00	12.00

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dose, the frequency of positive mutation and the number of viable colonies reduced.

3.2. Screening and selection

Colonies can be seen very clearly with the help of colony restrictor for their selection. Triton X-100 was used to curb fungal colonies in small size in the selection medium. More than106cells/ml were exposed to mutagenesis, which gave rise to 100 colonies (approximately) after dilution and culturing on PDA (supplemented with Triton X-100) as colony restrictor Triton X-100 was found useful due to small colony size and good round colonies. For further selection, the mutant colonies with larger zone were preferred. Triton X-100, a synthetic surfactant, can decrease bacterial and fungal growth. It results in the delaying of the logarithmic growth phase of microbes.

3.3. Decolourization by mutants

After mutagenesis, spores were inoculated on the Petri plates for maximum growth and inoculum was prepared for each mutant. Following inoculation, the triplicate flasks for each mutant with each shade strength were run along with each shade's control for 15 days in an orbital shaker (Fig. 4.2). After 15 days, all the flask were harvested to determine the enzyme activity (Table 4.2). The fabric was removed and washed thrice. K/S value of each sample was determined to calculate the percentage decolorization. It can be clearly seen from (Table 2) and (Fig. 5) that Mutant UV-60 with shade strength 0.5% showed highest percentage of decolorization. The percentage stripping done by the mutant UV-60 is 89%. Enzyme profile of mutant along with control which is parent strain also showed highest enzyme activity by mutant UV-60 (Table 3).

3.4. Pilling

The very complex property of textile fabrics is pilling, and the degree of pilling of fabric was evaluated by comparing the test specimen with visual standards. A scale ranging from 5 to 1 was used to evaluate the resistance to pilling employing the Martin dale Tester, and the results are depicted in Table 4. The bursting strength of biologically striped cotton fabric was 889, 874 and 890 Kpa for 0.5%, 2% and 4% shade strength, respectively.

3.5. Decolourization by selected mutant

All the UV treated mutants and parent strain of *Ganoderma lucidum* were cultured to decolourize dyed cotton fabric. It was observed that almost all the mutant strains decolorized the dye fixed on fabric by the production of enzymes more than that of the parent strain, but the mutant treated for 60 min was the best dye decolorizer.

3.6. Physical parameters optimization to enhanced decolorization by mutant UV-60 using RSM

Mutant UV-60 was selected for further optimization of results. The effect of independent variables like temperature, pH, incubation time and inoculum size were examined on the decolorization fabric (response) produced by the UV-60 mutant of *G. lucidum*. Five levels of each variable were studied in triplicate for a 30 runs design. The response (decolorization) was calculated at the end of the experiments. Mean triplicate trials were used to calculate response values. The optimum decolorization was seen at inoculum size 3 mL, incubation time 12 days; pH 4 and temperature 30 °C. The maximum decolorization obtained was 81.89% while minimum was 24.23%(Table 5).

Table 2
% Decolorization by all the mutants of *Ganoderma lucidum.*

Shadestrength (%)		Un-stripped	Biological stripping		
		Color strength (K/S value)	Color strength (K/S value)		Stripping (%)
			Control	treated	
Mutant UV-30	0.5	4.7 ± 0.1	4.2 ± 0.1	0.19 ± 0.1	85.31
	2	7.9 ± 3.2	6 ± 1.2	0.45 ± 2.8	70.54
	4	18.5 ± 1.59	15.11 ± 9.1	3.5 ± 11	62.75
Mutant UV-60	0.5	4.7 ± 0.1	4.2 ± 0.1	0.001 ± 0.1	89
	2	7.9 ± 3.2	6 ± 1.2	0.29 ± 1.2	72.27
	4	18.5 ± 1.59	15.11 ± 9.1	3 ± 0.7	67.27
Mutant UV-90	0.5	4.7 ± 0.1	4.2 ± 0.1	0.45 ± 0.1	79.78
	2	7.9 ± 3.2	6 ± 1.2	0.75 ± 0.8	66.45
	4	18.5 ± 1.59	15.11 ± 9.1	5 ± 0.9	54
Mutant UV-120	0.5	4.7 ± 0.1	4.2 ± 0.1	1 ± 0.10	68
	2	7.9 ± 3.2	6 ± 1.2	1.8 ± 0.11	53.16
	4	18.5 ± 1.59	15.11 ± 9.1	6 ± 0.16	49.04
Mutant UV-150	0.5	4.7 ± 0.1	4.2 ± 0.1	3 ± 0.6	25.65
	2	7.9 ± 3.2	6 ± 1.2	3.8 ± 0.14	27.84
	4	18.5 ± 1.59	15.11 ± 9.1	8.9 ± 0.21	33.56

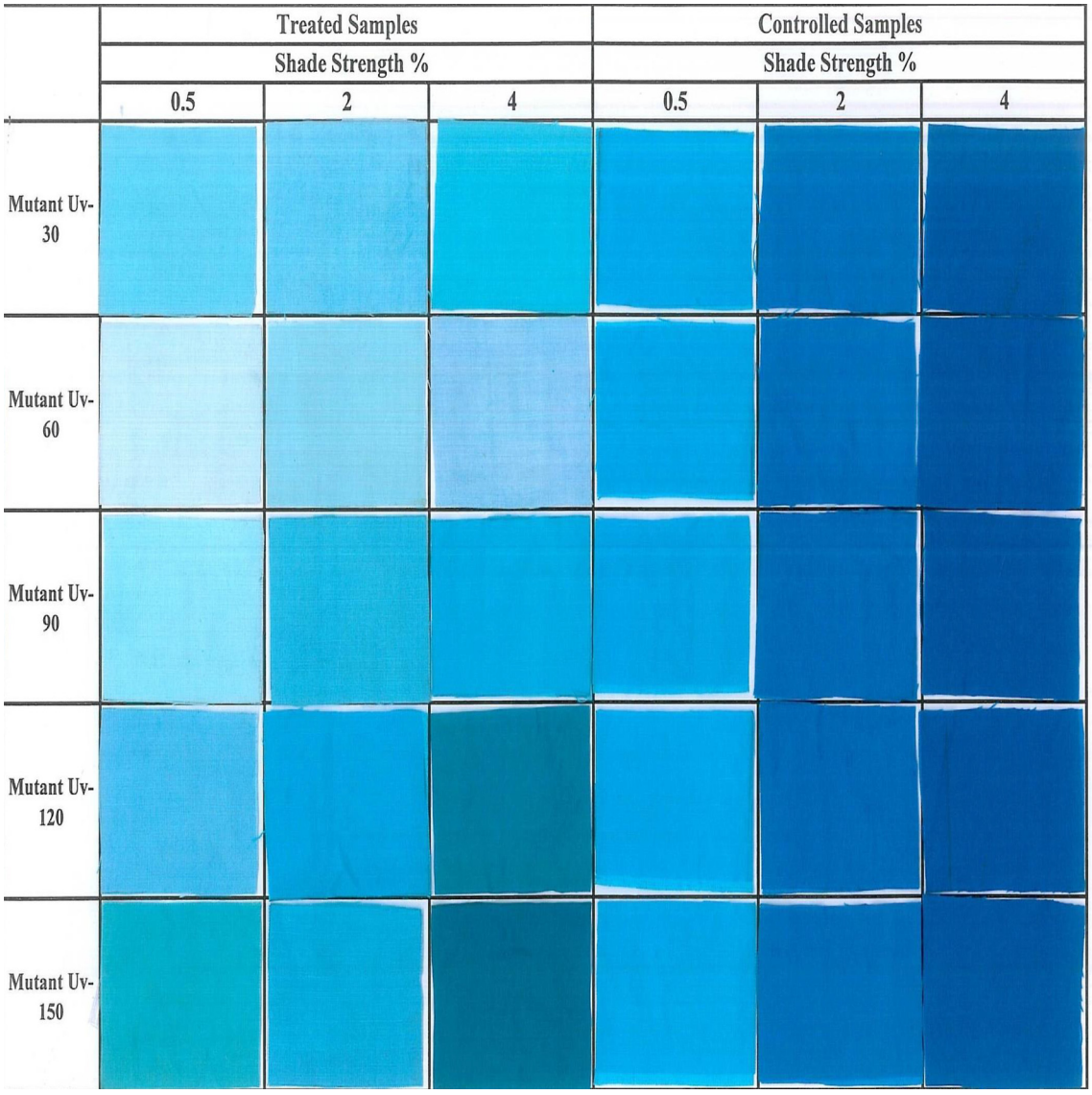

Fig. 5. Samples of cotton fabric dyed with Reactive Blue 21 in different shade strengths stripped by Ganoderma lucidum under optimum conditions.

Table 3
Profile of Enzymes synthesized by different mutants for stripping of cotton fabric of each shade strength.

	LiP			MnP			Lac		
Shade strength	0.50%	0.02	0.04	0.005	0.02	0.04	0.005	0.02	0.04
Control	693 ± 6.21	700 ± 2.9	698 ± 1.3	344 ± 1.10	342 ± 3.5	340 ± 1.0	59 ± 0.4	59.8 ± 2.1	56 ± 0.9
Mutant UV-30	737 ± 10	740 ± 3.1	731 ± 0.3	352 ± 1.9	355 ± i.7	351 ± 1.2	63.19 ± 0.3	62 ± 1.0	64 ± 0.1
Mutant UV-60	785 ± 0.09	788 ± 3.0	796 ± 0.4	427 ± 5.1	430 ± 1.2	427 ± 4	75.75 ± 2.6	77 ± 3.6	73 ± 2.3
Mutant UV-90	730 ± 5.5	716 ± 3.2	721 ± 2.7	400 ± 6.8	389 ± 0.1	395 ± 0.1	62.7 ± 2.8	62 ± 2.6	59 ± 1.1
Mutant Uv-120	691 ± 4.0	650 ± 2.6	675 ± 4.1	301 ± 5.1	305 ± 3.2	303 ± 0.05	56 ± 3.5	54 ± 00	58 ± 1.0
Mutant UV-150	429 ± 1.3	413 ± 1.1	411 ± 6.1	219 ± 1.7	216 ± 1.7	215 ± 4.3	43.25 ± 2.3	45 ± 0.2	40 ± 0.1

Table 4
Pilling resistance ranking of biologically stripped Fabric.

Untreated	Biologically stripped sample	Control for stripped sample
0.5%	4	4/5
2%	4	4
4%	4	4/5

3.7. Result of ANOVA

The value of adjusted R^2 will be smaller than R^2 if a small number of factors are used in the model with a minimal sample size. The close values of adjusted R^2 to actual R^2 value in decolorization indicated that the linear, square and interaction terms could illustrate 90.02% of the variation. Representations of the process by the model were satisfactory. Lower values of coefficient of variation 0.90% revealed the accuracy and reliability of experiments performed. The results of the ANOVA are presented in Table 6. Predicted values of the F test and P values with the factors were taken. The smaller the P-value is, the better the importance of the corresponding coefficient. Non-significant lack of fit is suitable for the model to fit (see Table 7).

Table 6
ANOVA for decolorization of dyed fabric by UV-60 mutant in Response Surface Model.

Model Terms	Value	Model Terms	Value
Mean	52.56	Adj R-Squared	0.8071
Std. Dev	9.16	R-Squared	0.9002
Press	4125.64	Adeq Precision	8.798
C.V.	17.43	Pred R- Squared	0.7728

3.8. Effect of variables

The optimum pH for different fungal strains was 4–6, depending on the components present. It was noticed that the optimum temperature range of 30-35 °C is the best range for decolorization. In our case, 3 mL (medium level) of inoculum showed maximum decolorization. Our results proved that 10 –15 days are optimum for fermentation for attaining maximum decolorization.

The current project was planned to investigate the potential of different mutant strains of *Ganoderma lucidum* to decolorize the dyed cotton fabric. Screening experiments were conducted to select the best mutant strain for maximum decolorization. *Ganoderma lucidum* was irradiated by UV irradiation mutagenesis for a different period to generate the mutants with enhanced enzyme production and the highest decolorization of dye set on cotton fab-

Table 5
Central Composite Design for optimization of decolorization by UV-60 mutant of *Ganoderma lucidum*.

Runs	A: pH	B: Temperature (°C)	C: Inoculum size (mL)	D:Fermentation time (hours)	Response Colour Stripping (%)
1	3.00	20.00	5.00	15.00	25.32
2	4.00	30.00	3.00	9.00	79.81
3	3.00	20.00	1.00	15.00	24.23
4	4.00	30.00	3.00	9.00	79.65
5	4.00	30.00	3.00	6.00	72.09
6	3.00	40.00	1.00	3.00	35.06
7	4.00	30.00	3.00	9.00	77.4
8	3.00	40.00	1.00	15.00	52.34
9	5.00	20.00	5.00	15.00	27.76
10	3.00	40.00	5.00	15.00	52.21
11	4.00	30.00	3.00	9.00	80.01
12	5.00	40.00	5.00	15.00	50.43
13	3.00	20.00	1.00	3.00	32.21
14	4.00	35.00	3.00	9.00	67.91
15	5.00	20.00	1.00	15.00	27.9
16	4.00	30.00	4.00	9.00	78.44
17	5.00	20.00	1.00	3.00	27.06
18	3.50	30.00	3.00	9.00	37.15
19	3.00	20.00	5.00	3.00	30.33
20	4.00	25.00	3.00	9.00	40.65
21	4.00	30.00	3.00	9.00	79.91
22	5.00	40.00	1.00	15.00	48.45
23	5.00	40.00	5.00	3.00	43.01
24	4.00	30.00	3.00	9.00	64.03
25	3.00	40.00	5.00	3.00	45.33
26	4.50	30.00	3.00	9.00	70.09
27	4.00	30.00	2.00	9.00	76.72
28	5.00	40.00	1.00	3.00	42.91
29	5.00	20.00	5.00	3.00	27.48
30	4.00	30.00	3.00	12.00	81.89

Table 7
ANOVA for Response Surface Quadratic Model.

Source	Sum of Squares	Df	Mean Square	F value	p-value prob > F	
Model	11,352	14	810.86	9.67	**<0**.0001	Significant
A	12.64	1	12.64	0.15	0.7034	
B	1572.53	1	1572.53	18.75	0.0006	
C	9.58	1	9.58	0.11	0.7401	
D	55.09	1	55.09	0.66	0.4304	
AB	0.19	1	0.19	0.00228	0.9635	
AC	3.05	1	3.05	0.036	0.8512	
AD	0.53	1	0.53	0.00631	0.9377	
BC	10.13	1	10.13	0.12	0.7331	
BD	150	1	150	1.79	0.0233	
CD	2.33	1	2.33	0.028	0.8698	
A^2	535.04	1	535.04	6.38	0.2011	
B^2	486.37	1	486.37	5.8	0.0294	
C^2	254.93	1	254.93	3.04	0.1017	
D^2	225.11	1	225.11	2.68	0.1222	
Residual	1258.21	15	83.88			
Lack of fit	1062.8	10	106.28	2.72	0.8164	Not significant
Pure Error	195.41	5	39.08			
Cor total	12610.2	29				

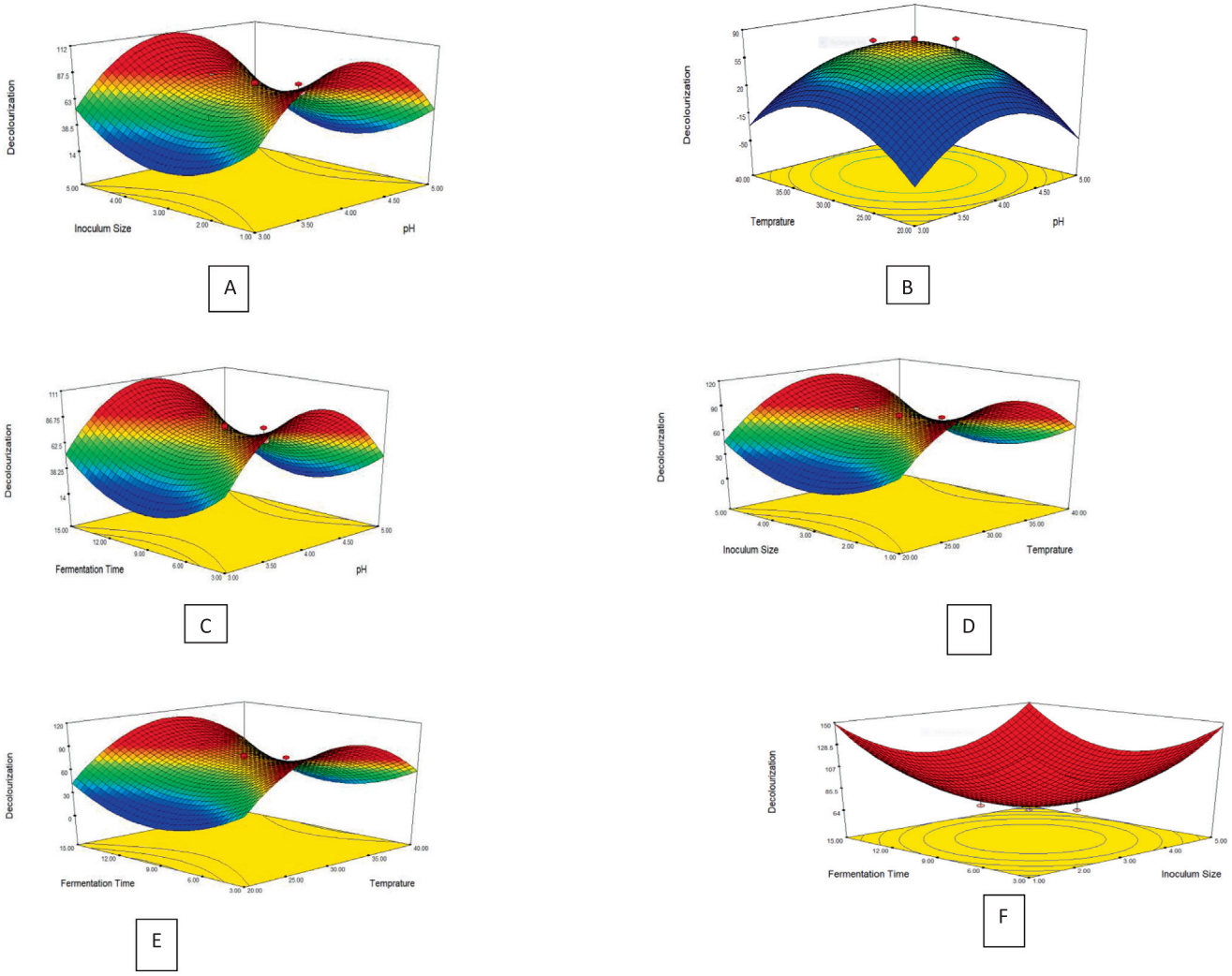

Fig. 6. A, pH vs temp. B pH vs Inoculum size, C pH vs fermentation, D temp. vs inoculum, Etemo. vs fermentation, F inoculum size vs fermentation.

ric. Triton X-100 was used as a colony restrictor after mutagenesis to hamper fungal colonies in minute size. Every mutant strain, along with control, was used for dye decolorization. After 15 days of incubation, mutant UV-60 was selected as the best decolourizer of dyed fabric.

Different physical conditions like temperature, pH, fermentation time and inoculum size were optimized through RSM (Response Surface Methodology) for the highest dye removal. Thirty runs were employed to optimise these four variables by using a Central Composite Design (CCD). This design helps analyze the results of variables interaction on the rate of decolorization and in the determination of optimum conditions. The optimum decolourization conditions were pH 4; temperature, 30 °C; inoculum size, 3 mL; fermentation time, 12 days.

After determining the regression coefficient (R^2), the mutant's value was 0.9002, indicating the model has good fitness for experimental data. The variation (CV) coefficient for the mutant quadratic polynomial model was 17.4 signifyingfies that the model was fit for experimental data. 3D and Contour surface graph were made for the mutant strain to illustrate the interactive influence of a wide range of variables on dye removal (Fig. 6).

4. Discussion

All the mutant strains along with control was used to decolorized the dye. After 15 days of incubation mutant UV-60 was selected as the best decolorizer of dyed fabric. The parent strain of *G. lucidum* showed 64% of decolorization of dyed fabric. This enhanced the need of mutation and after mutation it was cleared that the mutants of *G. lucidum* depicted higher decolorization level than of parent strain. It was predicted by our results that the effect of pH is obvious and the rate of decolorization increased with the initial increase in pH up to 4 and then started to decrease as pH value exceeded 4.5. High pH may affect the decolorization due to some conformational changes in the enzymes behind this decolorization (Tavares et al., 2006). Optimum pH for different fungal strains was 4–6 and it depends on the components present in the decolorizing dye. Response surface analysis showed that pH in range 3.0–5.0 has significant effect on decolorization (Kapdan and Kargi, 2002). The optimized pH for decolorization fall in range of 3.5–4.5.

Temperature is also an important factor which play vital role in maximum decolorization. White rot fungus is capable of forming enzyme under optimum conditions. It was noticed that optimum temperature range 30 °C–35 °C is best optimum range for decolorization. The results showed that at 30 °C *G. lucidum* showed better performance for stripping the colour from dyed cotton fabric. Maximum colour stripping (89%) was achieved at this temperature. Optimum temperature for the growth of most WRF and activities of their ligninolytic enzyme is around 30 °C. Optimum pH and temperature are crucial factors for maximum decolorization.

The growth of microorganism in liquid fermentation medium is also influenced by inoculum size. Low inoculum size is not suitable for growth of microorganism while large quantity of microorganism cause competitive inhibition (Sabu et al., 2005). Large quantity of inoculum results in lowered metabolic activities of microorganism due to lack of nutrients. In our case the medium level of inoculum 3 mL showed maximum decolorization.

Fermentation time is also an important factor which affects the biosynthesis of fungus. The percentage of stripping increased with longer reaction period indicating the direct relationship between the percent stripping and decolorization. The rising capacity in rate of decolorization was prominent up to 10 days after which very little increase in percent colour stripping was observed. *G. lucidum* mutants required 9–15 days to attain highest decolorization of dye. Our results showed that the optimum fermentation time for maximum decolorization was 10–15 days. Comparison between different variable could be evaluated by Fig. 6. Our current results suggest that response surface methodology is a unique and advance technique that not only considers one or more factors at a time but it also determines the interaction among different factors and their significant effect on increased decolorization in less time.

5. Conclusion

The mutant strain UV-60 showed 89% decolorization of dye under optimum temperature, pH, fermentation, and inoculum size. Enzyme activity assay showed that UV-60 produced LiP (785 U/mL), Mnp (427 U/mL) and Lac (75 U/mL) enzymes during color stripping of fabric. Lip was the primary enzyme that played a role in fabric decolorization, as this stripping depends on the enzyme system, so it caused no damage to the fabric. Various quality tests, including bursting strength and pilling, showed no harmful impact of biological stripping on the fabric's quality. The results of the study revealed the possibility of using *Ganoderma lucidum* as an environmentally friendly, efficient and low cost medium for color stripping of cotton fabrics dyed with Reactive Blue 21.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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