Distressing of Denim Using Laccase From *Bacillus Tequilensis* SN4

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Abstract

Denim is the most preferred outfit for men, women and kids in the world. Distressed denim is currently in trend due to increasing market demand. Industrial bleaching of denim for distressed look results in the generation of huge environmental pollution. In the present study, an economical and eco-friendly process has been generated for the bleaching of denim fabric using a *Bacillus tequilensis* SN4 laccase. The treatment condition was standardized and it was observed that 15 $lUml^{-1}$ of laccase with 1mM ABTS at 63°C, pH 7.8 resulted in maximum decolorization of denim. Furthermore, the recycling of ABTS was also carried out by adding fresh enzyme and denim piece and repeating the mediator molecule. The results obtained suggested that after 4 cycles with the same mediator, same level of decolorization was achieved. This results in decreasing the overall cost of the process while distressed fabric is generated without causing any pollution.

Introduction

Textile manufacturing is one of the world's biggest and oldest industries with India standing world's second-largest garment and apparel manufacturer position. Among the different textile industries, the denim segment especially the blue denim has always worn the crown as one of the most preferred outfit. The global denim business is predictable to inflate at a composite yearly growth rate of over 6.5 percent from 2015 to2020, with a market size of $113 billion to $153 billion.10% of overall denim manufacturing market is shared by India.

Indigo dye is commonly used for dyeing denim fabric. The sap of *Indigofera tinctoria* shrub was used for the isolation of natural indigo. However, synthetic indigo obtained by reacting aniline with formaldehyde, hydrogen cyanide, sodium hydroxide and water are subsequently being used in current denim market (Munoz et al., 2016). Indigo has very low affinity for cellulose fibers. It is also insoluble in water and is usually fixed to the fabric through a complex oxidation-reduction reaction. Alkaline reagents like sodium dithionite and hydroxyacetone are used to reduce the Indigo to leuco enolate which is soluble in water. This reduced form of indigo has great affinity for cellulose fibers resulting in deep penetration of dye in the fabric. Upon oxidation, indigo returns to its insoluble form resulting in binding to the fabric (Munoz et al., 2016).

Distressed denim is currently in trend due to increasing market demand. To give denim distressed look, denim is usually washed with pumice stones in washing machines in the presence of strong oxidizing reagents like sodium hypochlorite (Juciene et al., 2006). However, using stones can cause fabric wear and tear, as well as a problem with the environmental disposal of grit created by the stones (Couto, 2012). Physical removal of pumice stones and dust particles from garments and machines is needed which results in high labour costs. Big, costly laundry machines are often harmed by the stone-washing process.

Furthermore, to fully remove the stones from denim, it must be washed multiple times. Hypochlorite used for denim bleaching is a sturdy oxidizing agent which also attack cotton and reduce its strength (Couto, 2012). Moreover, stone washing process discharges large amount of waste water containing dye (Wambuguh and Chianelli, 2008). Furthermore, the hypochlorite process is extremely harmful to the environment, both because of the chlorite itself and the subsequent neutralisation steps, which produce large quantities of salts, causing disposal and contamination issues such as an increase in biological oxygen demand (BOD) and chemical oxygen demand (COD) in effluent, as well as an increase in effluent processing cost (Yavuz et al., 2014).

Enzymatic processing of denim has potential to replace the stone washing procedure for denim finishing (Campos et al., 2001). Because of natural cellulosic fibers in jeans, the use of cellulase has been proposed to give denim the stone wash look (Cavaco-Paulo, 1998). However, certain amounts of cellulose fibers are removed from the surface during enzymatic treatment, causing damage to the fabric. Treatment with cellulases also leaves marks of back-staining on denim due to redeposition of indigo released by enzymatic treatment. The jeans are rigorously cleaned with surfactants to prevent such unwanted re-coloration of threads. This process can cause unwanted colour fading in jeans, as well as increased water consumption during the washing process (Juciene et al., 2006). Moreover, the indigo dye removed is discharged into the effluent stream (Wambuguh and Chianelli, 2008).

Apart from cellulases, laccase have also been explored for the processing of denim (Couto, 2012). Laccases oxidizes indigo carmine specifically to isatin, therefore, no color is released during treatment (Yavuz et al., 2014) because of which there is no problem of back-staining. Use of fungal laccase have been reported for denim finishing in literature (Pazarlioglu and Foncu, 2005; Fu et al., 2012; Couto, 2012; Yavuz et al., 2014). However, as the dyeing of fabric is carried out at high pH and temperature conditions, fungal laccases have little use at industrial scale. Previously, we had reported an extracellular laccase from *Bacillus tequilensis* SN4 (Sondhi et al., 2015). SN4 laccase is highly stable at various industrial environment (Sondhi et al., 2014). Therefore, in this study, the ability of SN4 laccase to biobleach denim fabric was studied. The major problem in the utilization of laccase in denim bleaching is the cost incurred by the mediator molecule. In the present study, attempts have also been made to recycle mediator to reduce the cost involved in overall process.

Materials And Methods

Chemicals

Sigma Aldrich supplied all of the substrates (USA). Hi-media was used to buy various textile dyes.

Microorganism, laccase production Laccase Assay

Bacillus tequilensis SN4 was previously isolated in our laboratory (Sondhi et al., 2015). The culture was maintained on M162 medium as described by (Sondhi et al., 2015). Laccase was produced by submerged fermentation method (Sondhi et al., 2015). M162 medium containing 0.6% yeast extract, 300µM manganese sulphate and 300µM copper sulphate was inoculated with 0.3% of 24 h old culture of *Bacillus tequilensis* SN4 inoculum. Fermentation was carried out for 96 h and extracellular enzyme was extracted and purified (Sondhi et al., 2014). The enzyme assay was carried out for 5 minutes at 85°C with 2mM DMP as suggested by Sondhi et al. (2014).

Treatment of dyes with SN4 laccase

Different dyes were enzymatically treated with laccase with/without mediator. A total reaction volume of 10ml was prepared with 50g ml^{-1} (50 ppm) dye and 4 $IUml^{-1}$ SN4 laccase in a total reaction volume of 10ml. Mediator *viz.* HOBT, ABTS, and syringaldehyde was added at a final concentration of 1mM. Reaction was carried out at 65°C for 24 hours. Every dye's absorbance was measured at its respective wavelength after treatment. Percentage of decolorization was calculated as suggested by Sondhi et al. (2018).

Optimization of treatment condition for indigo carmine decolorization with SN4 laccase by one variable at a time (OVAT) method

As indigo carmine was degraded by laccase without any mediator, the effect of different treatment conditions *viz.* enzyme dose (0-10 $IUml^{-1}$), temperature (60-80°C), treatment time (0-10 h) and pH (6.0-9.0) on indigo carmine (50ppm) degradation was optimized by OVAT method. For pH 6.0 and 7.0, 0.1M phosphate buffer was used. For pH 8.0 and 9.0, 0.1M Tris-HCl was used and for pH 10.0, 0.1M carbonate-bicarbonate buffer was used. Conditions optimized in each reaction were kept constant in subsequent reactions. Total reaction volume was set to 10 ml (pH 8.0, 0.1M Tris-HCl buffer). Appropriate substrate and enzyme control were prepared for each reaction. After treatment, absorbance was measured at 600nm. Percentage of decolorization was calculated as suggested by Sondhi et al. (2018).

Statistical optimization of parameters for the decolorization of indigo carmine by RSM based desirability method

Using statistical software response surface methodology(version 9.0.4.1), decolorization of indigo carmine were further standardised. Four factors were optimised at α value of ±2: enzyme dosage, time, temperature, and pH (Table 1). In each experiment, the response was measured as a percentage reduction in colour. The 30-trial experimental plan looked at the four variables at five different levels: low (-2, -1), medium ((0)), and high (+1, +2). Analysis of variance was used to test the response data.

Table 1 Experimental range and levels of independent test variables used in central composite rotary design

Codes	Factors	Units	-2	-1	0	+1	+2
A	Enzyme dose	$IUml^{-1}$	1	3	5	7	9
B	Time	h	0	2	4	6	8
C	Temperature	°C	55	60	65	70	75
D	pH	-	5.0	6.0	7.0	8.0	9.0

Prediction of optimum values

After obtaining the model equation that explains the operation, the numerical optimization option of the programme was used to optimise decolorization. The independent variables were kept within the experimental setup's range, and the response was set to the limit. The independent variables' expected levels and the maximum response were used to construct a solution.

Validation experiments

To test the statistical model, an experiment was performed under the model's optimal conditions, and the response (percentage decolorization) was measured and compared to the expected values. Each experiment was repeated three times, and the results were presented as mean ± SD.

Indigo carmine (50ppm) was treated under conditions optimized by RSM. In substrate control, dye treatment was carried out under same conditions without enzyme and in enzyme control, indigo dye was excluded from reaction mixture. After treatment, percentage decolorization was calculated as per equation 1.

Biobleaching of Denim using SN4 laccase

As SN4 laccase decolorized the indigo, its utilization for finishing of denim to give it shaded appearance was explored. For this, a uniform colored jean was taken from market and cut into pieces of sizes 4 x 4 cm^2. The fabric was treated under conditions optimized for indigo degradation i.e. 6.55 $IUml^{-1}$ of enzyme, pH 7.8, at temperature 63°C without and with 1mM HOBT or syringaldehyde or ABTS as mediator (from a stock solution of 10mM). Reaction mixture without enzyme and without/with mediator was taken as control respectively. The flasks containing fabric was incubated at 150 rpm in water bath shaker. Total reaction volume was 50 ml and treatment was carried out for 48 h. Jean pieces without enzyme were taken as control. After the treatment, the fabric was taken out from the flask and washed to remove the residual enzyme. Pieces were dried in an oven and observed visually for decolorization.

Optimization of denim finishing with SN4 laccase

The conditions for maximum decolorization of denim fabric were optimized with respect to parameters *viz.* enzyme dose (0-20 $IUml^{-1}$), treatment time (0-48 h) and ABTS concentration (0-2.0mM, from a stock solution of 10mM). Total reaction volume was 50 ml and treatment was carried out. Reaction mixture without enzyme but with mediator was taken as control. After the treatment, the fabric was taken out from the flask and washed to remove the residual enzyme. Pieces were dried in an oven and observed visually for decolorization.

Optimization of recycling of mediator

To optimize the recycling of ABTS, the denim fabric was treated under optimized conditions obtained in previous experiments i.e. 15 $IUml^{-1}$ laccase 65°C for 24 h in the presence of 1mM of ABTS as mediator. Total reaction volume was made to 50 ml. After each 24h, fresh fabric and enzyme was added to a final concentration of 15 $IUml^{-1}$ in the same flask but without any fresh mediator. The cycle was repeated 5 times. Reaction mixture without enzyme but with mediator treated under same conditions was taken as control. After each treatment, the fabric was taken out from the flask and washed to remove the residual enzyme. Pieces were dried in an oven and observed visually for decolorization.

Results

Different synthetic textile dyes were treated with 4 $IUml^{-1}$ of SN4 laccasewith/without different mediators i.e. HOBT, ABTS and syringaldehyde.. Results are listed in Table 2.

Table 2
Decolorization of synthetic textile dyes with SN4 laccase

Types of dyes	Percentage decolorization (laccase)	Percentage decolorization (laccase + HOBT)	Percentage decolorization (laccase + ABTS)	Percentage decolorization (laccase + syringaldehyde)
Azo dyes				
Sudan IV	4.84	15.18	29.75	2.96
Methyl red	15.19	12.58	17.71	10.4
Methyl orange	15.68	16.37	15.19	2.95
Trypan Blue	2.28	3.17	78.53	3.39
Congo red	8.45	11.38	63.56	7.17
Rhodamine B	7.23	13.51	7.07	6.28
Triphenylamine dye				
Malachite green	73.24	72.88	68.70	38
Indigoid				
Indigo carmine	92.38	93.37	94.25	38.36
Arylmethane dye				
Auramine O	17.86	18.25	26.07	2.36
Crystal violet	5.28	5.38	30.81	1.28

It was observed that various dyes were degraded to varying extent by SN4 laccase (Table 2). Degradation of some of the dyes was more effective in the presence of one or the other mediator; ABTS being the most and syringaldehyde, the least effective mediator. However, malachite green and indigo carmine were degraded even in the absence of mediator. As indigo carmine is an important dye with respect to its application in denim industry, its degradation was standardized further.

Optimization of degradation of indigo carmine with laccase treatment by OVAT method

Decolorization of indigo carmine was standardized in terms of parameters *viz.* enzyme dose, treatment time, temperature and pH by varying one factor at a time keeping the other constant. Conditions optimized in previous experiment were used in subsequent reactions.

Enzyme dose

A laccase dose of 5 $IUml^{-1}$ resulted in maximum decolorization (94.75%) of the indigo dye. Further increase in enzyme dose did not significantly affect the color reduction. Therefore, 5 $IUml^{-1}$ of SN4 laccase was selected for optimal decolorization (Fig. 1A).

Incubation time

The effect of treatment time on reduction in color of indigo dye was determined for a period of 0–10 h. Percentage decolorization of indigo increased linearly upto 5 h (96.38%). Further incubation did not result in a significant decrease in the color of the dye. Therefore, 5 h was selected as optimum time for decolorization (Fig. 1B).

Temperature

The effect of temperature on reduction in the color of indigo dye by SN4 laccase was assessed in the range of 60–80°C (Fig. 1C). It was found that the optimum temperature for laccase performance was 65°C at which 97.05% of decolorization was achieved.

pH

Effect of pH on indigo carmine degradation was studied. It was observed that SN4 laccase could effectively decolorize the indigo in the alkaline range and maximum decolorization was observed at pH 8.0 (97.57%). Therefore, pH 8.0 was selected for optimal decolorization of indigo carmine (Fig. 1D).

Thus, 5 $IUml^{-1}$ of SN4 laccase resulted in 97.57% decolorization of indigo carmine at 65°C, pH 8.0 in 5 h.

Optimization of enzymatic treatment of indigo by statistical methods

Central composite design was employed to study the interactive effect of variables *viz*. enzyme dose, reaction time, temperature and pH on decolorization of indigo carmine (Table 3). In 30 experiments, decolorization ranging from 0–95.81% was achieved (Table 3).

Table 3
Central composite design matrix with actual and predictive values

Run order	Enzyme Dose ($IUml^{-1}$)	Reaction time (h)	Temperature (°C)	pH	Actual value (% decolorization)	Predicted value (% decolorization)	Residual (% decolorization)
1	5	4	65	5	45.26 ± 0.11	46.28	-1.02
2	3	2	60	8	34.95 ± 0.02	33.68	1.27
3	3	6	60	8	79.81 ± 0.24	79.88	-0.07
4	1	4	65	7	39.29 ± 0.13	40.76	-1.47
5	7	6	60	8	95.81 ± 0.75	95.09	0.72
6	3	6	70	6	52.27 ± 0.25	50.47	1.80
7	7	6	60	6	62.26 ± 0.15	64.14	-1.88
8	7	2	70	8	55.95 ± 0.25	54.43	1.52
9	3	6	60	6	58.29 ± 0.28	57.25	1.04
10	7	2	70	6	58.24 ± 1.18	58.36	-0.12
11	7	2	60	8	39.25 ± 0.39	41.24	-1.99
12	7	6	70	8	91.50 ± 0.38	89.52	1.98
13	5	0	65	7	0.0 ± 0.0	1.13	-1.13
14	5	4	65	7	90.81 ± 0.25	90.68	0.13
15	7	6	70	6	77.35 ± 1.04	76.06	1.29
16	5	4	65	9	63.65 ± 0.58	64.98	-1.34
17	5	4	65	7	90.85 ± 0.26	90.68	0.17
18	3	2	60	6	26.26 ± 0.49	28.44	-2.18
19	3	6	70	8	55.54 ± 0.24	55.61	-0.07
20	5	4	55	7	72.28 ± 0.49	70.86	1.42
21	9	4	65	7	73.02 ± 0.25	73.91	-0.89
22	3	2	70	6	42.26 ± 0.59	40.42	1.84
23	5	4	65	7	88.81 ± 1.28	90.68	-1.87
24	3	2	70	8	29.85 ± 0.48	28.17	1.68
25	5	4	65	7	92.89 ± 0.29	90.68	2.21
26	5	8	65	7	63.81 ± 0.56	65.03	-1.22

Run order	Enzyme Dose ($IUml^{-1}$)	Reaction time (h)	Temperature (°C)	pH	Actual value (% decolorization)	Predicted value (% decolorization)	Residual (% decolorization)
27	5	4	65	7	90.25 ± 0.26	90.68	-0.43
28	7	2	60	6	30.29 ± 0.14	27.67	2.62
29	5	4	65	7	90.45 ± 0.26	90.68	-0.23
30	5	4	75	7	73.50 ± 0.44	77.27	-3.77

Model equation

An explanatory model was devised by the software using results of Table 3. Following equation was built to explain the association between percentage decolorization and the four important parameters:

$$\% \text{ decolorization} = +90.68 + 8.29A + 15.97B + 1.60C + 4.68D + 1.91AB + 4.68AC + 2.08AD - 4.69BC + 4.35BD - 4.37CD - 8.34A^2 - 14.40B^2 - 4.15C^2 - 8.76D^2$$

…Eq. (2)

Where,

R = response (percentage decolorization),

A = enzyme dose;

B = reaction time;

C = temperature;

D = pH respectively.

ANOVA for response surface methodology

Table 4 displays the model's study of variance (ANOVA). The linear, interactive, and square words all had an important impact on the decolorization of effluent water, as shown by the p-values of 0.0001. The lack of fit p-value was 0.0801, suggesting that this complete quadratic model fit the data well. The R^2 (0.9976) determination coefficient showed that the expected and experimental values were completely in sync. The modified R2 (0.9959) indicated that the independent variables were responsible for 99.59 percent of the variance in the answer, with the model accounting for just 0.041 percent of the total variation.

Table 4: Analysis of variance (ANOVA) for response surface model developed

Source	Sum of Squares	df	Mean Square	F-value	p-value prob>F	
Model	17596.87	14	1256.92	258.03	< 0.0001	significant
A	1648.05	1	1648.05	338.33	< 0.0001	1648.05
B	6124.81	1	6124.81	1257.35	< 0.0001	
C	61.70	1	61.70	12.67	0.0029	
D	524.67	1	524.67	107.71	< 0.0001	
AB	58.52	1	58.52	12.01	0.0035	
AC	349.88	1	349.88	71.83	< 0.0001	
AD	69.31	1	69.31	14.23	0.0018	
BC	352.13	1	352.13	72.29	< 0.0001	
BD	302.24	1	302.24	62.05	< 0.0001	
CD	306.25	1	306.25	62.87	< 0.0001	
A2	1905.97	1	1905.97	391.27	< 0.0001	
B2	5686.39	1	5686.39	1167.35	< 0.0001	
C2	472.90	1	472.90	97.08	< 0.0001	
D^2	2105.45	1	2105.45	432.22	< 0.0001	
Residual	73.07	15	4.87			
Lack of Fit	64.40	10	6.44	3.72	0.0801	not significant
Pure Error	8.66	5	1.73			
Cor Total	17669.94	29				
Model fitting	C.V= 3.55%	R^2 = 0.9959	R^2 (pred) = 0.9783	R^2 (adj) = 0.9920		

A: Enzyme Dose; B: reaction time; C: Temperature; D: pH

Interaction between variables

Figure 2 depicts the relationship between two variables when one is held at its optimum value. It was observed from the 3-D plots that increasing the enzyme dose and pH upto a central value resulted in increased decolorization of indigo carmine, however, increase or decrease in temperature did not have much significant effect on decolorization. With increase in time, decolorization rate increases upto 5 h and then decreases.

Optimization of the Mathematic Model

The numerical optimization option of the software was used to predict levels of the four parameters with the target of "maximizing" reduction in color. For optimum decolorization (99.08%), predicted parameters were enzyme dose 6.55 $IUml^{-1}$, 63.11°C temperature, 5.7 h reaction time and 7.83 pH. Enzymatic treatment of indigo was done under predicted conditions (Table 5) in triplicates which results in maximum reduction of 99.24% in color (Fig. 3) which was very close to the predicted one (99.02%).

Table 5: Validation of predicted model

Variables					Response (% reduction in color)			
Std run	Laccase ($IUml^{-1}$)	Time (h)	Temperature (°C)	pH	Actual	Predicted	Residual	Error (%)
1	6.55	5.7	63.11	7.83	99.24 ± 0.34	99.08	0.16	0.16

Values represent mean ± SD (n=3)

Application of laccase for denim finishing

Treatment of denim with SN4 laccase

Treatment of dark blue colored denim was done with 6.55 $IUml^{-1}$ SN4 laccase at 63°C, pH 7.8 (0.1M Tris-HCl buffer) for 48 h. It was observed that although laccase was able to decolorize indigo dye without any mediator, no decolorization of fabric was observed under these conditions (Fig. 4).

Treatment of denim in the presence of mediators

Denim treatment was repeated with SN4 laccase in the presence of 1mM HOBT/syringaldehyde/ ABTS as mediator for 48 h. Use of different mediators resulted in different level of contrast. However, maximum decolorization of denim fabric was observed in the presence of 1mM ABTS (Fig. 5).

Optimization of enzyme dose for denim biobleaching

To standardize the enzyme dose, denim fabric was treated with 0–20 $IUml^{-1}$ of laccase in the presence of 1mM ABTS as mediator at pH 7.8, temperature 63°C for 48 h (Fig. 6). Maximum decolorization was observed with 15 $IUml^{-1}$ of SN4 laccase. After this not much change was seen in color.

Optimization of time for denim biobleaching

For the standardization of optimum time for denim decolorization, the treatment of fabric with 15 $IUml^{-1}$ of SN4 laccase was carried out at 63°C for 0–48 h in the presence of 1mM ABTS as mediator. It was observed that maximum level of decolorization could be achieved after 24 h of treatment (Fig. 7). Further, treatment did not result in increase in decolorization. Therefore, 24 h was selected as optimum time for decolorization.

Optimization of ABTS concentration

To standardize the ABTS concentration, the ABTS was added in a final concentration of 0-2mM from 10mM stock. Denim fabric was treated with 15 $IUml^{-1}$ of SN4 laccase at 63°C, pH 7.8 for 48 h. It was observed that maximum decolorization was achieved with 1mM ABTS (Fig. 8). Further, increase in ABTS did not have any significant effect on decolorization. Therefore, 1mM concentration of ABTS was selected as optimum. A separate control was set for each concentration of the mediator, no decolorization was observed in any of the control (Data not shown).

Optimization of recycling of ABTS for denim finishing by SN4 laccase

To make the process economical, recycling of ABTS (by the addition of fresh enzyme) for the use of denim finishing with SN4 laccase was standardized. The fabric was treated with 15 $IUml^{-1}$ of laccase for 24 h at 63°C, pH 7.8. After each 24 h, fresh fabric and enzyme, to a final concentration of 15 $IUml^{-1}$, was added in the same flask but without any fresh mediator. The cycle was repeated 5 times. It was observed that after 4 cycles with the same mediator, same level of decolorization was achieved (Fig. 9). Addition of fabric and enzyme 5th time resulted in decreased level of decolorization.

Discussion

Textile dyes released in effluent water creates lots of environmental pollution. Therefore, degradation of harmful dyes and disposal of industrial effluent is always been a major problem for the industry. Laccase is an enzyme which has the capability to degrade and decolorize textile dyes efficiently. Therefore, in this study, first the degradation ability of SN4 laccase towards different dyes was studied. Industrial effluents typically contain 10-50ppm of dye (Ghaly et al., 2014); therefore, 50ppm concentration of each dye was evaluated for decolorization purposes. Different synthetic dyes were treated with 4 $IUml^{-1}$ of SN4 laccase in the presence and absence of different mediators *viz.* ABTS, HOBT and syringaldehyde. Out of the three mediators tested, ABTS was found to be the best mediator. Moilanen et al. (2010) and Fu et al. (2012) have also reported ABTS to be a good mediator for dye decolorization. In the presence of syringaldehyde, very low level of decolorization was observed. This might be due to the decrease in stability of SN4 laccase in the presence of syringaldehyde (Singh et al., 2019). Syringaldehyde has been reported to effect the stability of laccase in some other studies also (Galai et al., 2012).

It was observed that SN4 laccase was able to decolorize congo red (63%) and trypan blue (78%) in the presence of ABTS as mediator. Congo red and trypan blue are diazo dyes which are known to be carcinogenic (Zollinger, 2003; Lade et al., 2015). Degradation of these dyes is highly required to prevent damage to environment and human life. Extracellular laccase from *Bacillus* sp. ADR was unable to decolorize trypan blue and congo red (Telke et al., 2011).

Triphenylamine dye (malachite green) and vat dye (indigo carmine) were degraded by SN4 laccase without any mediator. Indigo is being used in industries to dye cotton fabric (Tian et al., 2013). The effluent released from such industry is highly colored due to the presence of residual indigo. Degradation of indigo carmine is highly recommended before disposal for saving environment (Wambuguh and Chianelli, 2008). Spore bound laccase from *Bacillus* sp. WD23 and *B. amyloliquefaciens* have been reported to decolorize indigo carmine by 70% and 85% respectively (Wang et al., 2011); but because of their spore bound nature, these laccases cannot be applied at industrial scale.

As SN4 laccase could degrade indigo carmine without any mediator, its degradation was standardized in detail with respect to parameters like enzyme dose, incubation time, temperature and pH. The conditions were first optimized by OVAT approach and then by statistical method using response surface methodology (RSM). After one variable at a time method (OVAT) approach, 97.57% decolorization of 50ppm indigo carmine was achieved with 5 $IUml^{-1}$ of SN4 laccase at 65°C, pH 8.0 in 5 h, without the use of any mediator. The decolorization rate increased to 99.24% after the optimization by RSM (6.55 Uml^{-1} SN4 laccase, 5.7 h reaction time, 63.11°C temperature and pH 7.83). Spore bound laccase from *Bacillus pumilus* could decolorize indigo by only 20% within 48h at pH 6.8 (Reiss et al., 2011).

As SN4 laccase could effectively degrade indigo, the ability of SN4 laccase to biobleach denim fabric was explored under conditions optimized for indigo degradation. It was observed that although SN4 laccase was able to degrade indigo carmine in solution without any mediator, no decolorization of denim fabric was achieved even with higher doses of SN4 laccase. This result is in agreement with the literature reports where no report exists in which laccase alone could decolorize denim (Solis-oba et al., 2008; Couto, 2012; Yavuz et al., 2014). This can be due to the insoluble nature of indigo on fabric. Indigo carmine is water insoluble, therefore, to dye cotton fabric, indigo is first reduced to water soluble leuco form, in which yarn is dipped and then oxidized to its insoluble form (Barfoed and Kirk, 1999). This insoluble form of indigo is accessible to the mediator compound not to the enzyme. Therefore, the role of the mediator is to mediate the transfer of electrons between oxygen and indigo (Sondhi et al., 2021).

Three different mediator compounds *viz.* HOBT, ABTS and syringaldehyde were screened for denim finishing by SN4 laccase. Only laccase + ABTS system was able to give required decolorization to the fabric. Decolorization was uniform and no problem of back-staining or spots were observed. Moreover, no color was released during treatment. Therefore, denim finishing with SN4 laccase is an environment friendly alternative to stone washing and cellulase treatment.

Further, the conditions of laccase treatment for denim finishing were optimized. Optimum decolorization of denim was achieved with 15 $IUml^{-1}$ at 63°C, pH 7.8 in the presence of 1mM ABTS in 24 h. The major constraint for industrial application of laccase mediator system is their high cost (Johannes and Majcherczyk, 2000). Solis-Oba et al. (2008) have reported that ABTS mediaor system can be recycled for the degradation of indigo. However, fresh enzyme has to be added to the reaction mixture because enzyme gets denatured by the active radicals generated during the reaction (Rehmann et al., 2014). Therefore, in the present study, the recycling of ABTS was studied by the addition of fresh enzyme after each treatment cycle of denim. It was found that ABTS accomplishes a recycling ability. The addition of fresh fabric and enzyme without new mediator resulted in same level of contrast after repeated cycling. To the best of our knowledge, no one in

literature has studied the recycling of ABTS for denim finishing. Even though ABTS is an expensive mediator, the fact that it can be reused to accomplish several cycles of laccase treatment of denim, makes it feasible to apply this process at industrial scale.

Conclusion

In the present study, ability of SN4 laccase in decolorzing denim was explored. The results suggested that SN4 laccase is highly suitable for its application in denim industry for replacing the traditional process of stonewashing as well as for the bioremediation of textile industry effluent containing indigo carmine. Furthermore, as the novelty of the process lies in the repetition of mediator molecule which no one has explored before. This reduce the overall cost of the process making the process industrially feasible.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

Fundings

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Authors' contributionss

SS performed all the experiments and has written the manuscript. AK and DK analyzed and interpret the data. NG is the corresponding author and responsible for data analysis and interpretation.

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Figures

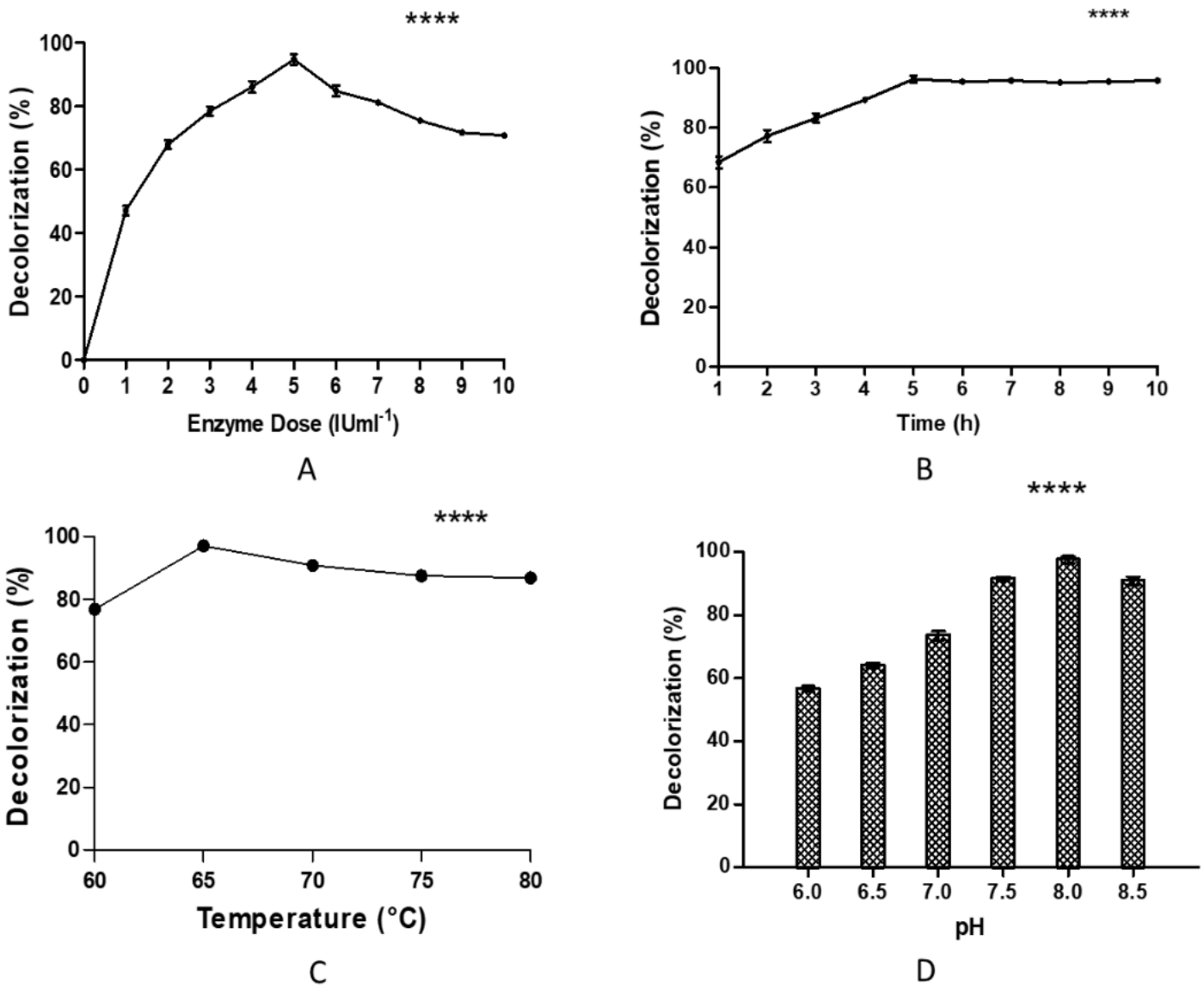

Figure 1

Effect of different conditions on decolorization of indigo carmine: A) SN4 laccase dose B) Values represent mean ± SD, n = 3; The asterisks indicated significant differences: **** $p \leq 0.0001$ analyzed by unpaired student's t-test

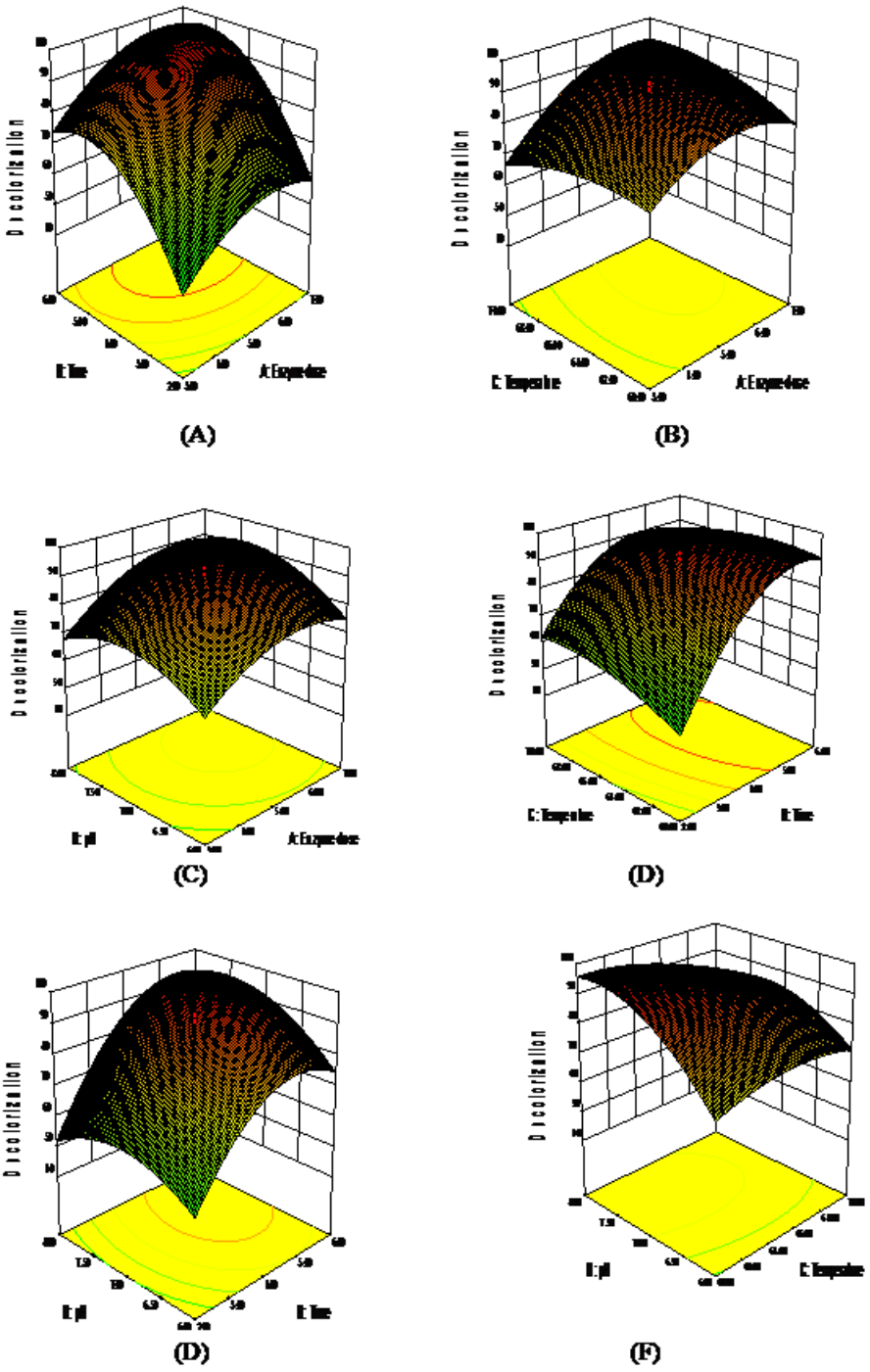

Figure 2

Depicts the relationship between two variables when one is held at its optimum value. It was observed from the 3-D plots that increasing the enzyme dose and pH upto a central value resulted in increased decolorization of indigo carmine, however, increase or decrease in temperature did not have much significant effect on decolorization. With increase in time, decolorization rate increases upto 5 h and then decreases.

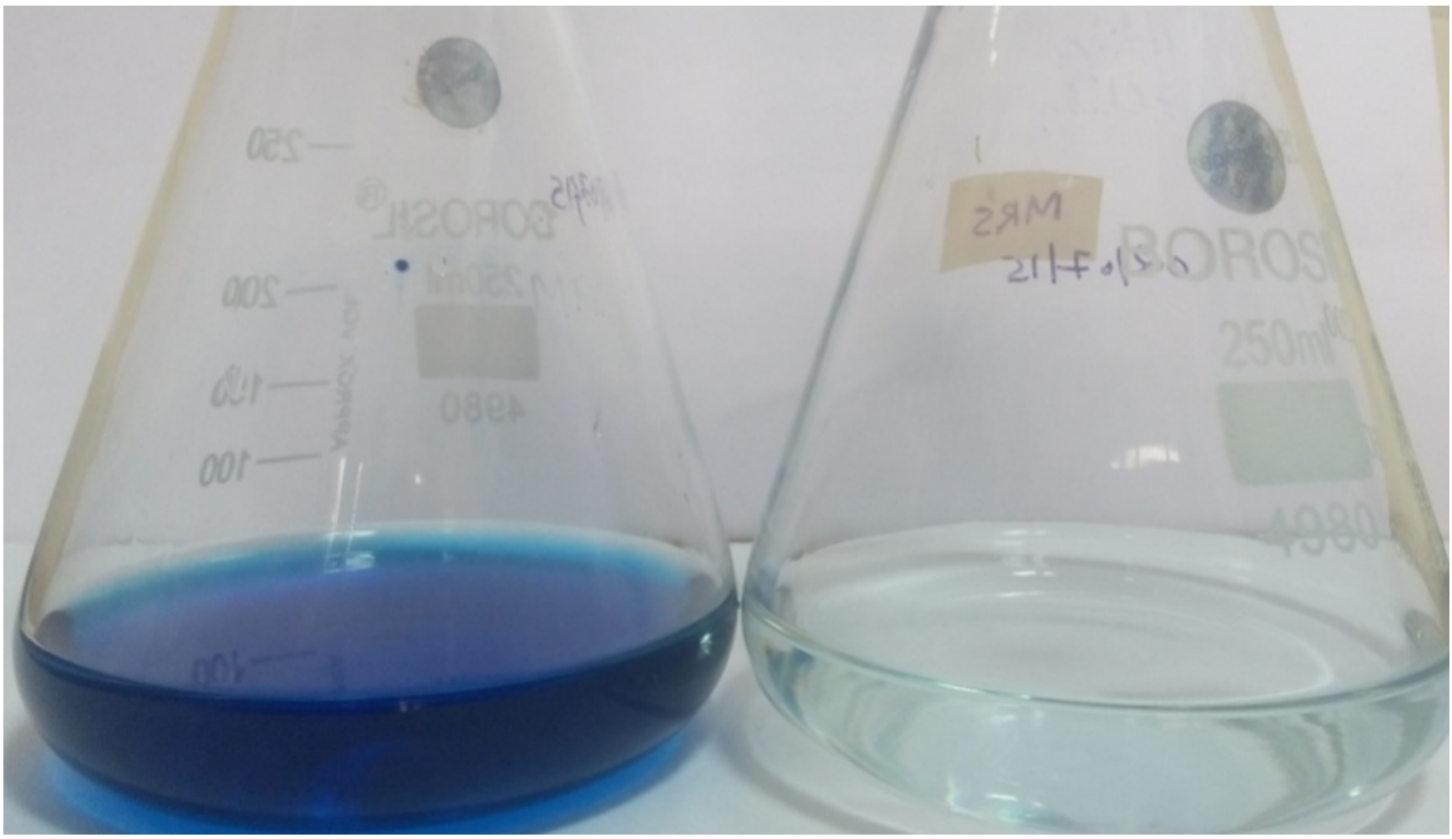

Figure 3

Decolorization of indigo carmine by SN4 laccase under optimized conditions

Figure 4

Denim finishing with SN4 laccase (A) untreated fabric (B) control (C) laccase treated

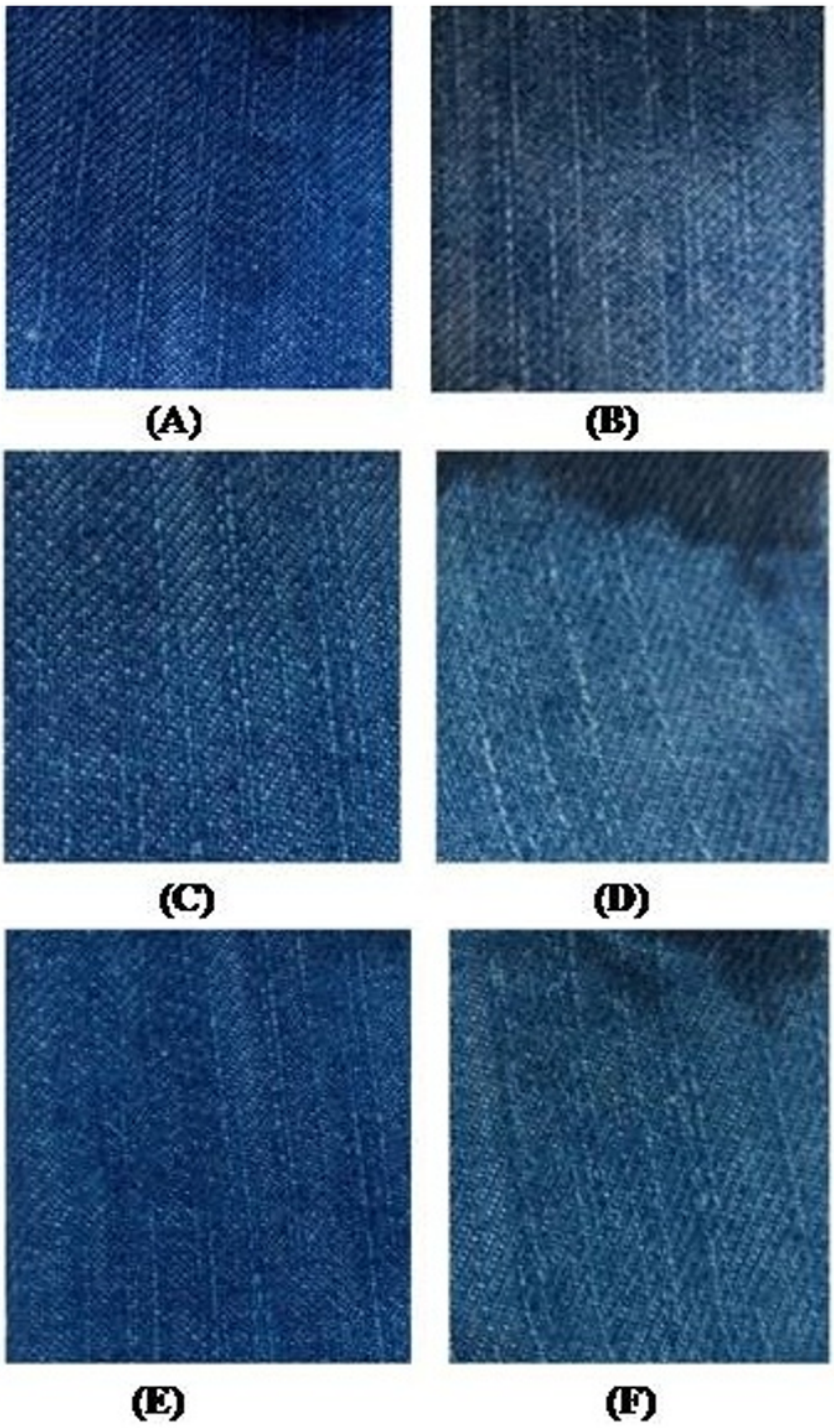

Figure 5

Denim finishing with laccase mediator system (A) Buffer + ABTS (B) Laccase + ABTS (C) Buffer +HOBT (D) Laccase + HOBT (E) Buffer + syringaldehyde (F) Laccase + syringaldehyde

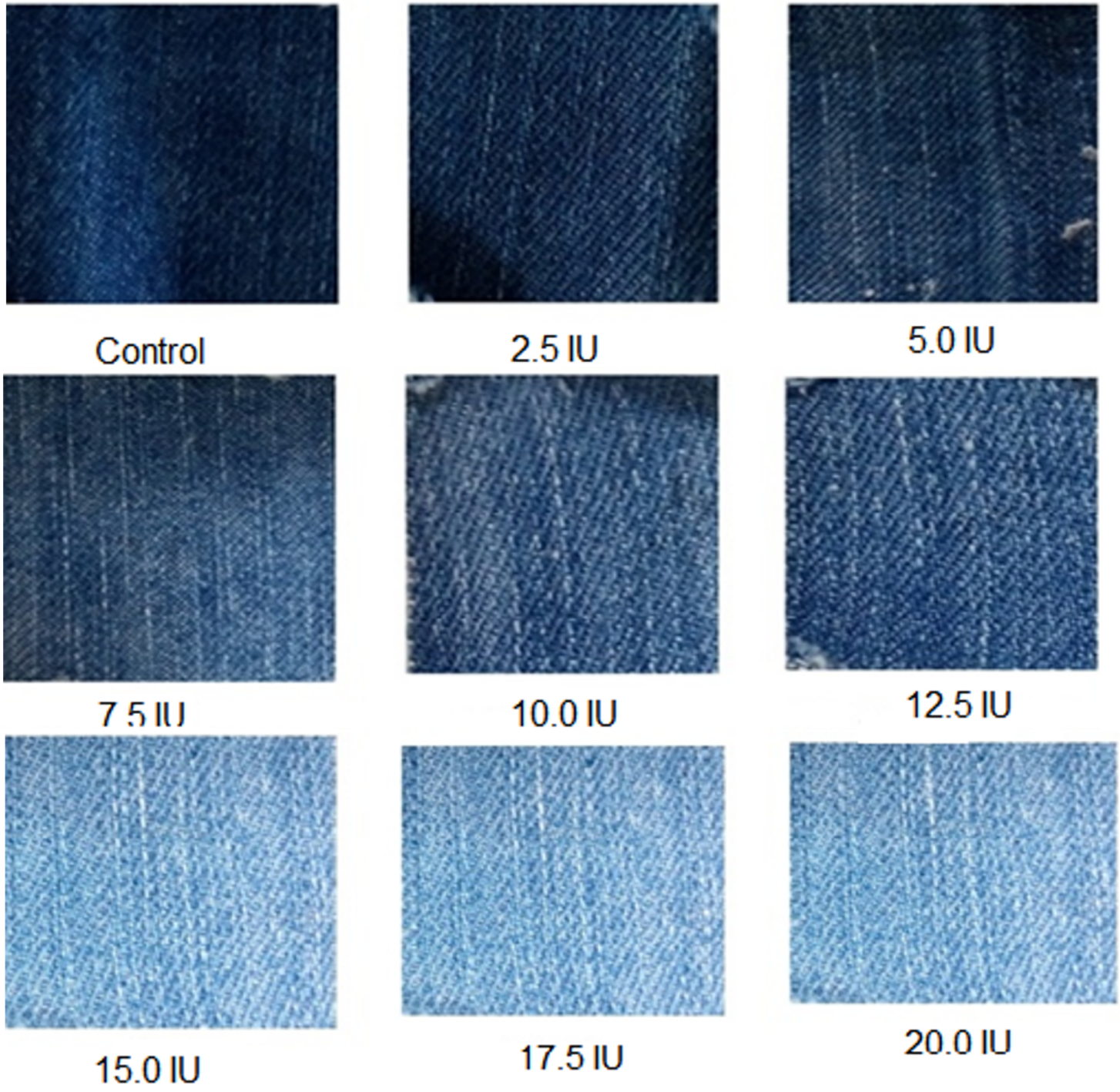

Figure 6

Effect of enzyme dose on denim biobleaching

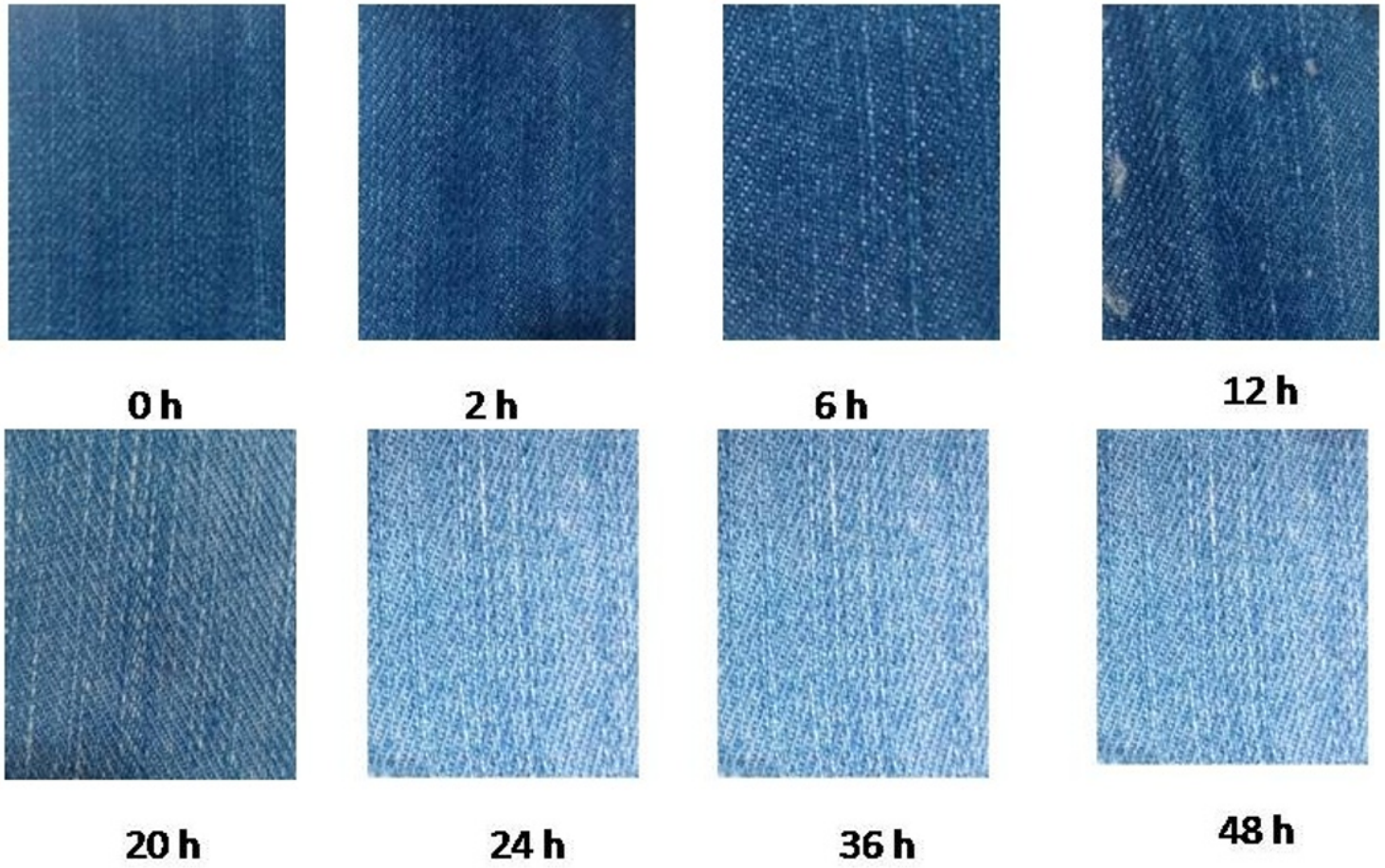

Figure 7

Effect of treatment time on denim biobleaching

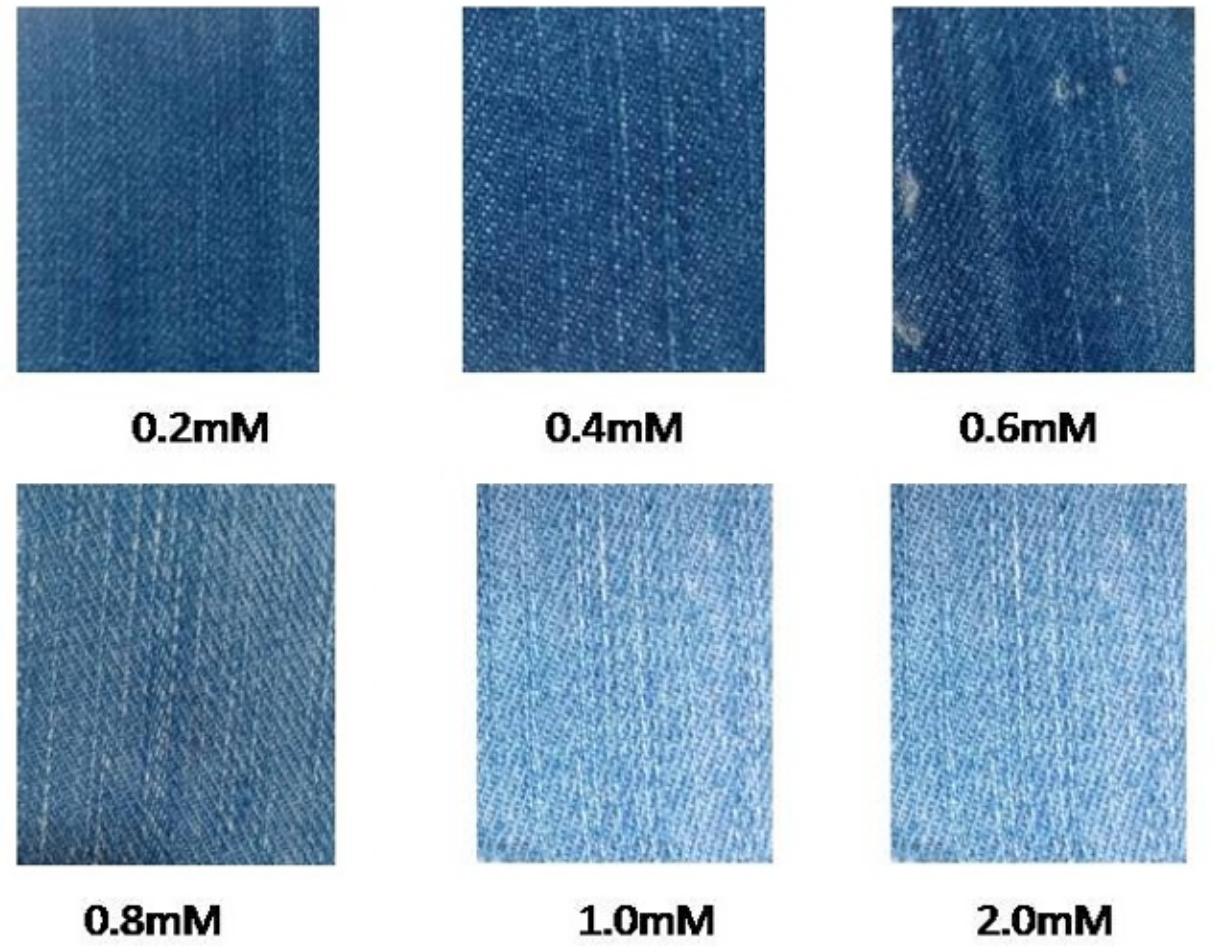

Figure 8

Effect of ABTS concentration on denim biobleaching

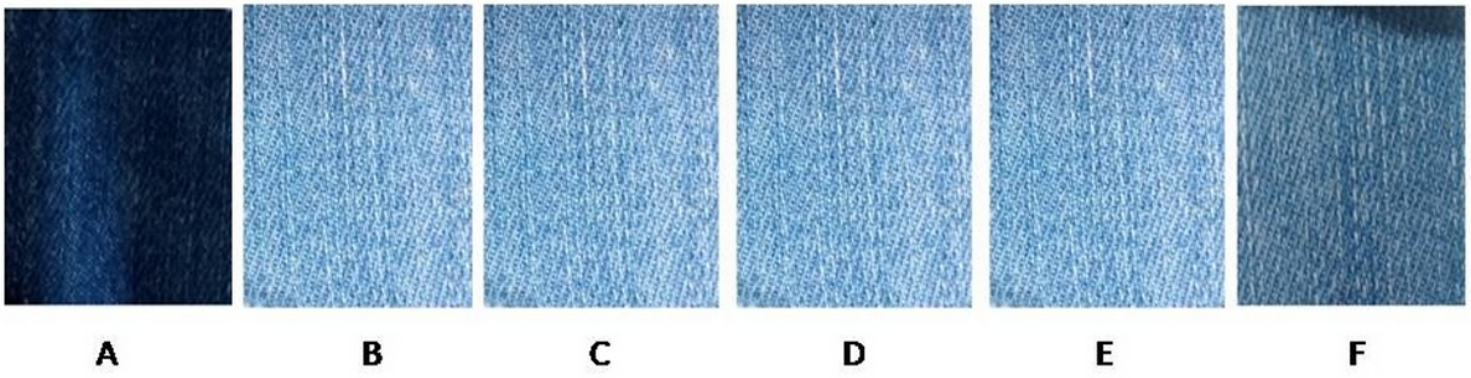

Figure 9

Effect of number of ABTS cycles of SN4 laccase treatment on denim biobleaching