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Enzymatic modification of cotton fiber for promising smart medical based material

Maharani Pertiwi Koentjoro
Study Program of Medical Laboratory Technology
University of Nahdlatul Ulama Surabaya
Surabaya, Indonesia
maharani@unusa.ac.id

Marisa Fitriana
Departement of Biology
Institute Technology of Sepuluh Nopember
Surabaya, Indonesia
marisa.fitriana03@gmail.com

Isdiantoni
Faculty of Agriculture
University of Wiraraja
Sumenep, Indonesia
antonie_isd@yahoo.co.id

Endry Nugroho Prasetyo
Departement of Biology
Institute Technology of Sepuluh Nopember
Surabaya, Indonesia
endry@bio.its.ac.id

***Abstract* — Color removal (bleaching) is an oxidation process whereby the undesirable inherent coloring component removal in organic material. Hydrogen peroxide (H_2O_2) is a fundamental stage of using bleaching agent in industrial and the medical field. As an oxidizing agent, H_2O_2 has merited a significant attention since it is able to degrade reactive dyes intensively. However, the H_2O_2 hydrolysis remain undesirable residues which leads a negative influence on fabric quality. Therefore, other alternatives method for bleaching is necessary by employing degradable materials like enzyme. Laccase is enzyme that considerable as a bio–bleaching agent for promising a smart medical based material. Therefore, in this study laccase from *Trametes versicolor* was used as bio–bleaching agents. The purpose of this study was to determine the potential of laccase for cotton fibers bio–bleaching. It was used H_2O_2 as control comparison meanwhile the laccase and laccase–ABTS (mediator) as treated groups. The brightness level and the presence of functional groups of cotton fiber were characterized by spectrophotometer and FTIR respectively. The highest brightness level of cotton fiber was obtained from laccase treatment as 85.68%. Laccase bio–bleaching showed aromatic functional groups attack which was confirmed by IR spectra. The result indicated that the cotton fibers bio–bleaching using laccase is an environmental friendly process with zero remaining toxicant oxidizing agent which is very promising for medical purposes.**

Keywords—Bio–bleaching, cotton fibers, laccase, Trametes versicolor

I. INTRODUCTION

Bleaching is an oxidizing process for removing dyes in organic materials [1, 2]. The bleaching process on textile fibers needs to be done to make the fiber color more uniform in the coloring process until the finishing process in the textile industry [1]. The most common natural fiber is cotton, which are consist of oils, fats, waxes, minerals and natural dyes [3]. Natural fibers and fabrics that have undergone a process of scouring still contain natural dyes that cause the color change to yellowish or brownish [4]. A change of yellowish or brownish color on cotton fibers is caused by flavonoids oxidation [5, 6].

Hydrogen peroxide as a conventional bleaching agent has applied under conditions alkaline [7]. The conventional bleaching process using hydrogen peroxide requires large amounts of water to neutralize the reaction [8]. However, conventional bleaching does not eliminate the yellowish color of cotton fibers perfectly [9]. The residual hydrogen peroxide still present in the cotton fibers also causes the process of cotton fibers staining to produce poor quality as a result [10].

Bio–bleaching process is an enzymatic technique to reduce the use of alkaline and more time–saving process [3, 11]. The enzymes that are widely used in textile bio–bleaching processes for instance laccase, manganese peroxidase, cellobiose dehydrogenase, and catalase [2]. Laccase (benzendiol: oxygen oxidoreductase, EC 1.10.3.2) is an oxidoreductase–class extracellular enzyme containing copper metal and capable of oxidizing some aromatic (diphenol, monophenol, aromatic amine) and non–aromatic compounds with radical catalyst–reaction mechanisms [12]. Laccase uses molecular oxygen to oxidize aromatic and non–aromatic compounds as a substrate and reduces it into water [13]. The good thing about laccase is that in the bio–bleaching process, it only requires molecular oxygen as acceptor electron meanwhile other oxidoreductases are also required other form acceptor electron, such as manganese and hydrogen peroxide. In the nature laccase can be found in insects, plants and microbes [14]. However, the fungi laccase has higher redox potential than other organisms (up to +800 mV) make this source of laccase is the most promising for industrial applications [15]. The fungi which are able to produce laccase cames from genus Trametes [12] with a wide area of applications such as the decolorization and detoxification of dyes and paper bleaching, phenol removal in wine processing, biosensors and synthesis of any organic compound [16].

Cotton fiber is mainly a natural polymer composed of cellulose [17]. The newly harvested cotton fibers have a high impurity content of natural pigments [5]. The removal of natural pigment impurities is usually done by conventional bleaching techniques that could damage cotton fibers molecular structure and requires high operational costs [2]. Therefore, in this study demonstrates the novel strategy of

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cotton fiber using laccase produced by *T. versicolor* to remove natural pigment impurities in moderate treatment conditions in order to avoid damage to the main polymer of cotton fiber. Accordingly, as a result the laccase treated cotton fiber could be a promising smart medical based material.

II. MATERIAL AND METHOD

T. versicolor was obtain from the Indonesian Culture Collection (InaCC), Center for Biological Research Institute of Science Indonesia. The strains have been tested for laccase producing ability through a spesific fermentation medium. The performance of laccase to cotton fiber phenolic oxidation was analyzed using spectrophotometric analysis, FTIR spectroscopy and cotton brightness test analysis.

A. Fungal maintenance and fermentation medium

Stock cultures of *T. Versicolor* were maintained by storing at 4°C in a cool cabinet and regularly weekly sub culturing on Potato Dextrose Agar (PDA) medium for 5–6 days at room temperature [7, 13]. Prior using for laccase production, the fungi cultures were then inoculated on GDP medium and incubated for 6–7 days at room temperature with shaking at 130 rpm.

B. Laccase production and enzyme preparation

The enzyme production was performed using a fermentation medium formulated by Fukushima and Kirk [18] and Nyanhongo [13]. *T. Versicolor* starter culture was added as much as 15 ml to fermentation medium and incubated at room temperature while shaking at 130 rpm. The laccase produced under fermentation medium were harvested based on the growth curve profile of *T. Versicolor* after the fungus enters the start of the stationary phase (day 9) [19]. After incubation, the liquid culture was filtered through Whatman No. 1 and centrifuged at 5,000 rpm for 15 min. The supernatants were carefully collected and used as crude enzyme extract for laccase assay (activity test, protein content and isoelectric point) [14, 20, 21].

C. Protein concentration assay, Laccase activity, and Isoelectric point

The concentration of protein was measured using the Bradford protein assay method [22] at 595 nm and Bovine Serum Albumin (BSA) as a standard.

The laccase activity test was performed using 2,2'–azino–bis (3–ethylbenzothazoline–6–sulfonate) (ABTS) as the substrate. The laccase activity test was conducted using the modified method [23]. Determination of laccase activity using ABTS were carried out by dissolving 0.5 ml of ABTS 0.1 mM, 0.01 ml of crude laccase extract, and 2.4 ml of 50 mM citrate buffer at pH 4.5. The reaction mixture was incubated at 35°C for 10 min. The ABTS standard curve was used for enzyme activity determination [20].

The isoelectric point (p*I*) of the laccase was determined by preparing 6 test tubes containing 1ml of enzyme solution and 1 ml of phosphate buffer with vary level of pH namely 3, 4, 5, 6, 7 and 8. The mixtures were vortex vigorously for 3 seconds. The time required for coagulating were recorded. Precipitation in the respective mixtures were used as isoelectric point value.

D. Cotton fiber bio–bleaching

For bleaching experiment, it was used five treatments, namely control (without treatment), H_2O_2 (30% commercial stock solution), laccase (1% v/v) laccase (2%v/v) and laccase (1%v/v) with mediator (ABTS).

The bleaching with H_2O_2 was carried out using the method reported by Osluoglu, and G. Arabaci [3] and Diller and Yang [24] with slight modification. Briefly, the cotton fibers were placed into the six Erlenmeyer flask containing 5%(v/v) of H_2O_2, 3%(v/v) of NaOH 0.2 M, and 3%(wt/v) of sodium silicate. The ratio between cotton fiber to the solution was set into 1:20. The bleaching process were carried out at 80–95°C for 30 minutes. After bleaching process, the cotton fibers were neutralized with 10% of acetic acid solution and rewashed one time with distilled water.

Bio–bleaching of cotton fibers using laccase was performed with 2 different laccase concentrations of 1%(v/v) and 2%(v/v). Bio–bleaching of cotton fibers was done according to Pereira et al [7] with slight modification. The experiments were initiated by heating the cotton fibers in boiling water for 15 minutes. Furthermore, the cotton fibers were washed with distilled water and let air dry for 7 h. The bio-bleaching reaction was started by putting 1 g of cotton fiber in each of two Erlenmeyer flasks containing 10 ml of citrate buffer pH 5.0, 5 ml of laccase 1% and laccase 2%, respectively. Meanwhile, the bio–bleaching of cotton fibers using laccase with ABTS were carried out the same way as bio–bleaching of cotton fibers using laccase above. One gram of cotton fibers was placed in the Erlenmeyer flask containing 5 ml of laccase, 2.5 ml of ABTS 0.1 mM and 10 ml of citrate buffer pH 5.0. Both of the bio-bleaching experiments were incubated at 37°C for 45 min. Finally, in order to terminate the enzyme reaction, the mixtures were added 20 ml of boiling water and let stand for 5 min. The treated cotton fibers then were washed 3 times with distilled water and again let it air dry for 7 h and stored in room temperature until used for the next analysis.

E. Analysis of residual pigments on enzymatic modified cotton fiber, FTIR (Fourier Transform Infrared Spectrocopy) analysis, and Cotton Fiber Brightness analysis

The estimation of cotton fibers pigment residue was measured according to the absorbance maxima at λ=510 nm using UV–Vis Spectrophotometer [25, 26]. The fibers were soaked in acetone prior analysis.

Cotton fibers before and after the bio–bleaching process were analyzed using FTIR Spectroscopy at wavelengths 400–4000 cm^{-1} to identify functional groups [27].

Further, the cotton fibers brightness was measured using Lorentzen & Wettre Elrepho Spectrophotometer Testing tool at a local paper print company PT Adiprima Suraprinta, Gresik–Indonesia.

III. RESULT AND DISCUSSION

A. The Production and characterization of T. versicolor laccase

Laccase production was carried out by growing the fungi *Trametes versicolor* in medium fermentation formulated by Nyanhongo et al [13] and Fukushima and Kirk [18]. The

fermentation medium was modified by the addition of copper (II) sulfate ($CuSO_4$) as inducer [14, 28]. CuSO4 is the constituent of the catalytic center of laccase, hence it is important for the synthesis of the enzyme.

Characterization of *T. versicolor* laccase includes the activity, protein concentration and isoelectric point. Laccase activities was achieved 1,400.07 U/ml. using ABTS as a substrate. ABTS is a heterocyclic non–phenolic compound that can be oxidized to a stable cation radical ($ABTS^+$) and ABTS dication ($ABTS^{2+}$) [28, 29]. The blue–green color of $ABTS^+$ cationic radical intensity is correlated to the laccase activity [29]. Protein concentrations in crude laccase were observed using Bradford reagents using BSA as a standard. The protein concentration at the end of laccase fermentation was achieved at 0.172 mg/ml of BSA equivalent.

The surface of a protein has a net charge and would be on zero at a certain pH called an isoelectric point [31]. In general, amino acids in proteins have different isoelectric points. Based on the isoelectric assay, laccase was precipitate at pH 4.0 and the results were in accordance with a study conducted by Bourbonnais et al [31] which stated that laccase produced by *T. versicolor* has an isoelectric point at pH 4.0. At the isoelectric points, there is no electrostatic force, so the enzyme has solubility lowest at the isoelectric point and coagulation occurs [31, 32]. Characterization of laccase demonstrates the use of rice straw and $CuSO_4$ in the fermentation medium serves as laccase inducer. Rice straw mainly consisted of lignin and *T. versicolor* uses as carbon source. The good thing about Laccase is that it uses only dioxygen to oxidize substrates as electron acceptor while other oxidoreductases (i.e. manganese peroxidases) required additional electron acceptor such as hydrogen peroxide for its oxidation [14].

B. Cotton fiber bio–bleaching

For comparison purposes, bleaching of cotton fibers was conducted using different bleaching agents, namely laccase, laccase with mediators ABTS and H_2O_2. The result of cotton fibers color after bleaching process by each bleaching treatment shows in Figure 1. The color change in cotton fibers is evident in the treatment of H_2O_2. The bleaching process occurs when the bond of the chromophore (dyestuff) is disconnected or destroyed. The oxidation process of flavonoid compounds in cotton fibers by H_2O_2 occurs at a pH between 10–12. In alkaline conditions, H_2O_2 dissociates to form ion hydroxyl. The mechanism of free radical formation in aromatic groups (chromophores) is carried out by the ion hydroxyl. Free radicals are formed due to the transfer of electrons to peroxide and then demobilization of electrons in double bonds lead decolorization [33].

As shown in Figure 1, the color of cotton fibers treated using laccase 2% is slightly brighter than the laccase–ABTS and laccase 1% treatments, whereas the color of cotton fibers with laccase–ABTS treatment is slightly brighter than the color in the 1% laccase treatment. This suggests that the laccase produced by *T. versicolor* is capable of oxidizing pigments in cotton fibers and in accordance with the statement of Pereira et al [7]. In other word, laccase produced by *T. versicolor* could oxidize the phenolic compounds of lignin present in the pulp and the decolorization of flavonoids in cotton. The brightness level analysis of cotton bleaching which are determining by the difference of brightness in each treatment is shown Figure 2.

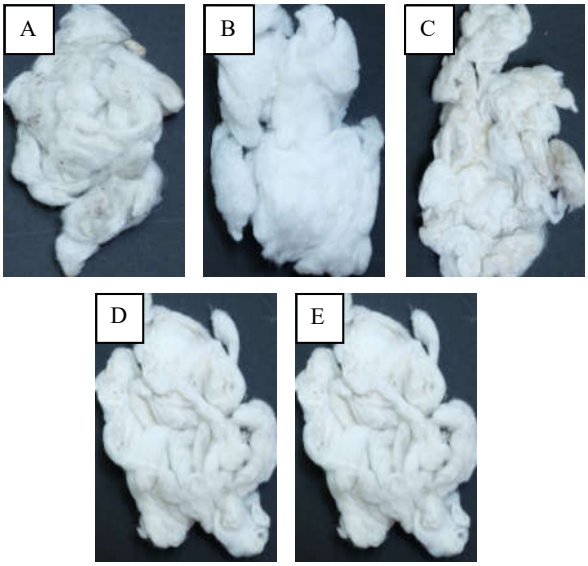

Figure 1. Bleaching cotton fiber visualization: (A) untreated cotton fiber (negative controls); (B) Cotton fiber bleached with H_2O_2 (positive control) (C); Cotton fiber bleached with Laccase 1% (D); Cotton fiber bleached with Laccase 2%; (E) Cotton fiber bleached with Laccase and ABTS.

Cotton fibers naturally contain color pigments of flavonoids that include phenolic compounds [35]. In the bio–bleaching process using laccase, the phenolic are oxidized to phenoxyl free radicals by attack the hydroxyl group of aromatic compounds. The phenoxyl free radicals is unstable and then re–oxidized again by laccase (e.g. forming quinone from phenol) or undergoing non–enzymatic reactions (polymerization) [37].

The use of laccase–ABTS as a bio–bleaching agent can also oxidize phenolic compounds in cotton fibers. The addition of a mediator (ABTS) aims to improve the oxidation process [36]. The addition of ABTS is to have higher oxidation of non–phenolic compounds [16], since laccase is mostly only oxidation of phenolic compounds [38]. The amount of oxidized substrate is increased with the addition of mediator [37]. Oxidation of compounds using laccase–ABTS is called the Laccase Mediator System (LMS) [39]. According to Jong–Rok et al [40], the oxidation capacity of phenol compounds in the bio–bleaching process using LMS is greater than using laccase. However, in this study, the brightness level of cotton fibers resulting from bio–bleaching using laccase–ABTS was lower than that of bio–bleaching using 2% laccase. This most probably because of some ABTS were still stick on the fibers.

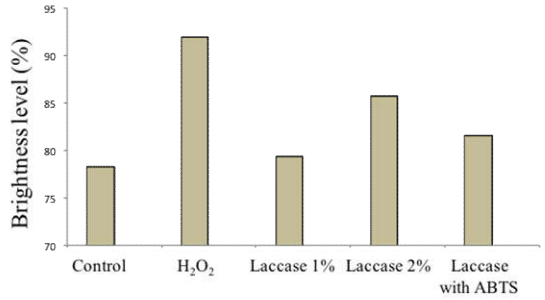

Figure 2. Brightness level of cotton fiber in the different treatment of bleaching agent.

The purpose of bleaching is to removed impurities from the fiber through removing the chromophore components that absorb light in the fiber and increase the whiteness level of fabric. The bleaching process can improve the optical properties of the pulp against light absorption, light scattering and reflections expressed in white [34]. Figure 2 shows that the highest brightness level is in the treatment using H_2O_2 (positive control) that is equal to 91.92%. The brightness level on laccase treatment 1%, laccase 2% and laccase–ABTS are 79,33%; 85.68% and 81.57%, respectively. The brightness level of cotton fibers resulting from bio–bleaching is higher when compared with treatment result using buffer (negative control). The use of laccase 2% as bio–bleaching agent yields cotton fibers with higher brightness level than using laccase–ABTS and laccase 1%. Brightness level shows the less–absorbing chromophore component. Based on research conducted by Pereira et al [7] pre–treatment of cotton fiber before bleaching process stated that the brightness level has increased significantly that is equal to 8,5% in cotton fiber with pre–treatment using laccase.

Spectrophotometric analysis was performed to determine the absorbance change of solution used before and after bleaching process. The results of the spectrophotometric analysis are shown in Figure 3. indicated that there is a change in the absorbance of the solution used in the bleaching process. The absorbance ratio of the solution shows that after the bleaching process there is an increase in absorbance caused by the presence of a compound derived from the cotton fiber oxidation process. The result indicated that the absorbance ratio of bleaching solution does not related with bleaching result. In bleaching process using H_2O_2 which is a positive control, the absorbance ratio value is lower when compared with the treatment using laccase. The low absorbance ratio in H_2O_2 treatment can occur because during the bleaching process the H_2O_2 is dissociates into H^+ and HO_2- [33].

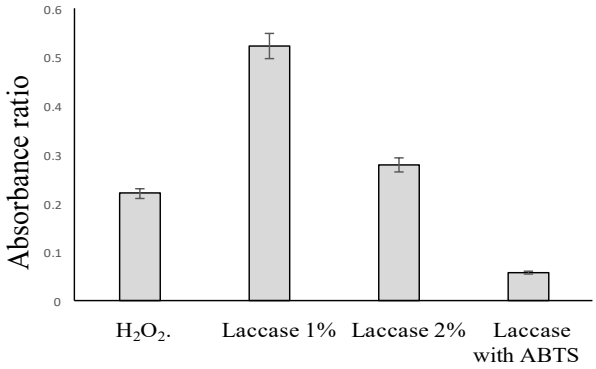

Figure 3. The ratio of the absorption residual cotton pigment after bleaching comparing to the control (λ = 510 nm)

C. FTIR Analysis of Cotton Fiber Bleaching Results

FTIR spectra of cotton fiber functional groups is seen in Figure 4.

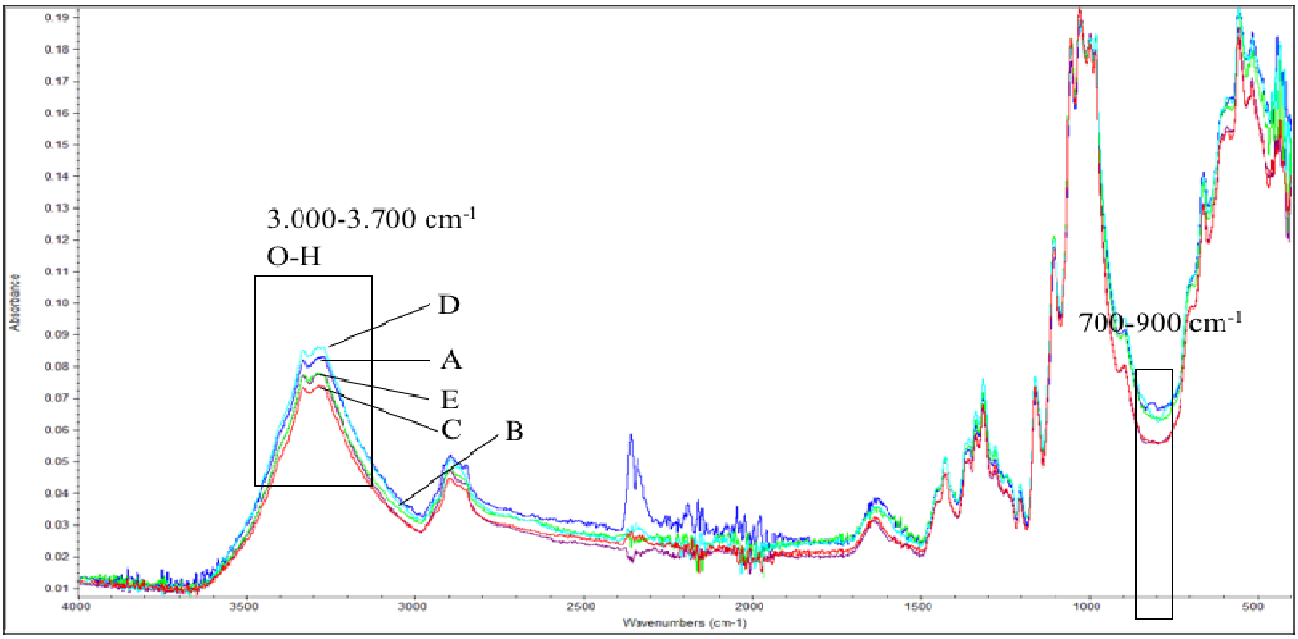

Figure 4. FTIR spectra of cotton fibers resulting in bio–bleaching using different agents: untreated cotton fiber (negative controls); (B) Cotton fiber bleached with H_2O_2 (positive control) (C); Cotton fiber bleached with Laccase 1% (D); Cotton fiber bleached with Laccase 2%; (E) Cotton fiber bleached with Laccase and ABTS.

The range of bleaching cotton fiber absorbance in FTIR spectra is at 400–4,000cm^{-1} wavelength. The absorbance range at the wavelength of 3,000–3,700 cm^{-1} shows the group vibration (O–H) contained in the cotton fibers. The absorption of clusters (O–H) on the control cotton fibers has an absorption intensity at a wavelength of 3,272.72 cm^{-1}. In cotton fibers treated laccase 1%, laccase 2%, laccase–ABTS and H_2O_2 peak shift with higher absorption intensity than control, at wavelength 3,288,52 cm–1, 3,288,52 cm^{-1}, 3,284,5 cm^{-1}, and 3,280.1 cm^{-1} respectively. According to Nugroho Prasetyo et al [23], the uptake of the (O–H) group becomes higher due to increased purity of the cellulose chain after the bleaching process. The hydrogen bond between the cellulose chains becomes stronger than the hydrogen bonds on the unbleached cotton.

The characteristic band at 700–900 cm^{-1} indicates the missing peak, the vibration of the C–H group of aromatic compounds which constitute the dyestuffs in the cotton fibers, namely on bleaching using H_2O_2, laccase or laccase–ABTS.

These results implicated that the biotechnological potential of laccase as bio–bleaching agents was assessed, confirming the capacity to decolorize of cotton fiber and to enhance undesirable inherent coloring component. By the enzymatic process, dyes decoloting is enviromentally–friendly and minimums remain residues. In addition, cotton fiber produced from modifying by laccase is less residual chemical, theferore its appropriate and suitbale for alternatively to medical based materials. Cotton fiber materials resulting bio–bleaching has potential for healthcare and medical product ranges from simple gauze, scaffolds for tissue culturing, bandage materials to a variety of prosthesis for permanent body implants and much more [41, 42].

Medical materials are primarily made of biology inert polymers, commonly used in fabrics and other materials that are in contact with skin. As example, in diapers, specialized polymer materials including cellulose, polypropylene, polyester, and polyethylene arranged in different layers are used to provide optimal absorption of urine and feces. As such understanding diaper materials and addressing myths about chlorine, latex, dyes, and other chemical additives in diapers is critical [43].

CONCLUSIONS

The conclusions of this study confirm that laccase produced by *T. versicolor* possessed a strong ability for to oxidize cotton fibers. The parameters including the brightness values were analyzed. Under optimal conditions, 2% laccase treatment has very efficient for bio–bleaching as the highest brightness level as 85.68%. FTIR spectra confirmed that bio–bleaching of cotton fibers using laccase destroys cation fiber functional groups. The effect of enzymes treatment could be explained in terms of selective oxidize of coloring component by the laccase used and better preservation of fibers under these peroxide bleaching conditions. Further studies will be carried out to evaluate the uptake fiber as alternatively to medical based materials.

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