

Biodeterioration of Cotton by Cellulolytic Fungi

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Abstract: Fungal deterioration is a major issue in garment mills; mainly it affects high cellulosic materials like cotton. This study empirically explains the saccharification of cotton by *Trichoderma sp.*, *Aspergillus sp.*, *Cladosporium sp.*, *Fusarium sp.*, and *Helminthosporium sp.* which were cultured and applied in cotton wool broth. Extracted enzyme suspension was assayed with filter paper and activity was analyzed by DNS reagent method. *Trichoderma sp.* and *Aspergillus sp.* depicts high cellulolytic activity of 33.08 and 32.52 FPU/ml respectively on raw cotton, in addition their saccharifying ability depicted 1.25 and 1.23%. Optimum temperature and pH for activity of each fungal enzyme also affirmed.

Keywords: biodeterioration, biodegradation, cellulase, fungi, cotton, fpase.

1. Introduction

Cotton is one of the mostly used clothing all over the world. Use of cotton fibers in clothing is dated since 5000 BC. Current production of cotton is about 25 million tonnes annually. Cotton plants are belongs to the *Gossypium* genera. Wool appears around the seeds, it consists about 94% of cellulose, and hygroscopic nature of cotton facilitates the colonization of fungi and bacteria. Biological deterioration of cotton fibers was recorded in many textile mills. In 1960 in UK the annual loss of cotton was reported as 110,000 tonnes. Reasons for the fungal infections are mainly soil and water contact on the material during the storage, transport and usage. And there are more possibilities for the spreading of airborne spores. Most cellulolytic fungi are belonging to division Ascomycota. They produce extra-cellular cellululase enzymes, which acts synergistically to break 1, 4- β - glucosidic bonds of the cellulose molecule. Addition to the enzymatic damage their metabolites give colouring on cloth such as black or grey spots. Moreover, acidic substances produced by microorganisms such as succinic, acetic, oxalic, lactic, citric, malic, and fumaric assists further deterioration. 10% humidity is sufficient for the active colonization of fungi. Cotton contains minerals that promote the microbial growth such as K, Na, Ca, Mg and microelements like Iron, Copper and Zinc. Moreover, presence of glucose, sulfates, phosphorus, nitrogenous substances and glycidols also advantageous to the colony. However, protection to the garment is hardly achieved by applying coatings such as approved chemicals and resins [1]. This work is to examine the ability of different fungal enzymes in deterioration of cotton fiber in addition the optimum temperature and pH for their activity also verified.

2. Methodology

2.1 Source of Fungi and Initial Culturing

Samples were collected from sawdust, straw dust and sprinkled soil (garden, beach, Mud). Decaying wood particles and decaying leaf collected from the surroundings. Samples were collected into sterile containers and stored separately. Potato dextrose agar medium was used to grow the initial cultures, where samples were cultured by streak plate method and sprinkle method.

2.2 Methods Of Inoculation

2.2.1 Streak plate method

In order to isolate the fungi this method is best in practice. Initial streak is made with the sample then all other streaks are continuum of the previous strike using separate sterile tooth peck for each strike.

2.2.2 Sprinkled method

This is more suitable for the soil samples and sawdust. Particles were sprinkled on the medium. Well-spaced sprinkled particles would result in separation of colonies. Finally, petri-dishes were sealed with Para film, labeled and inverted dishes were incubated in a dark place. Visible colonies were observed after 4-7 days (Figure 1).



Sawdust sprinkled Straw sprinkled
Figure 1: Inoculation of samples for the first time on PDA medium by sprinkle method

2.3 Media used for sub culturing

2.3.1 Selective water agar medium

To separate the fungi causing cellulose digestion, a medium consisting cellulose as a sole carbon source was prepared. For cellulose whatman no. 1 filter paper made of 100% cellulose was used. Water agar medium was prepared by dissolving 4g of agar in 250ml of distilled water. Autoclaved pieces of filter paper (1cm \times 1cm) were used for inoculation.

After pouring the water agar medium on petri dishes, paper strips were carefully placed on top of the agar bed by a sterile forceps. After the inoculation, sealed dishes kept for 4-7 days of incubation. To avoid bacterial growth, antibiotic was added to the medium. This made the medium more selective to fungi.

Table 1: Antibiotics and their concentrations that used in the culture

Antibiotic	Ampicillin	Tetracycline
In a ml of stock solution	50mg/ml	100mg/ml
Final concentration in a ml of water Agar medium	50µg/ml	100µg/ml

Table 1 depicts the amount of antibiotics that were added for the preparation of selective water agar medium. Subculturing continued until the pure culture was available.

2.4 Culturing fungi in liquid medium containing cotton wool and preparation of crude enzymes.

In order to extract the secreted cellulolytic enzymes by each fungal colony, it is important to culture them in a liquid medium. Cellulolytic basal medium (CBM) was chosen for this purpose. Cellulolytic basal medium (g /250ml in distilled water) was prepared [3].

Diammonium tartrate ($C_4H_{12}N_2O_6$) 1.5
 Potassium dihydrogen phosphate (KH_2PO_4) 0.25
 Yeast extract 0.02
 $MgSO_4 \cdot 7H_2O$ 0.15
 $CaCl_2 \cdot 2H_2O$ 0.0002

CBM medium was autoclaved and 10ml aliquots were transferred to sterile 20ml bottles.

Cotton wool strips of same amount were submerged into the CBM medium aseptically inside the laminar airflow. Samples which were previously obtained from pure culture were inoculated by streaking on the submerged cotton strip aseptically by sterile tooth peck. Always a control bottle was kept without inoculation. Caps of the bottles were loosely fitted to allow the adequate air exchange. All the bottles were incubated for 4 days at room temperature ($25^{\circ}C$). After the incubation, observations were made, and the liquid medium, which contains the crude fungal enzymes, was collected.

Each bottle contains the cellulolytic enzymes that were secreted as extra cellular enzyme by each fungus colony. At the time of isolation, which is after a period of incubation it is better to vortex in slow speed in order to ensure the distribution of enzymes all over the liquid medium. About 1ml of aliquot was taken by sterile micro pipet and placed into the 1.5ml sterile centrifuge tubes. Centrifugation was done at 12,000 r.p.m for 15 minutes. The resulted supernatant consists of proteins that are mostly the fungal crude enzymes. Centrifugation is important for the separation of fungal spores since spores could not be allowed in the later filter paper assay.

2.5 Filter paper Assay

International Union of Pure and Applied Chemists recommended filter paper assay (FPA) as the standard measure of cellulase activity. Enzymatic reactions often occur in the presence of buffer, which helps to keep the reaction environment stable. This is obtained by maintaining the ionic balance and the pH unchanged. 2ml 0.05M of Trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) buffer was

used with the crude enzymes and filter paper strips ($0.5cm \times 0.1cm$) were used as substrate.

0.1ml of crude enzyme of each fungus was added with 0.15 ml of Trisodium citrate dihydrate solution, while the pH was maintained at 4.8. Always a blank was maintained in one tube without adding any fungal enzymes. Instead, it was replaced by same volume of sterilized distilled water. Then the Whatman no. 1 filter paper strip ($0.5cm \times 0.1cm$) was added as the substrate. Each tube was then incubated in 500C shaking incubator running at 100 r.p.m [2].

2.6 Measuring the activity of cellulolytic enzymes

The activity of extracted fungal enzymes can be measured quantitatively. Here the concentrations of reducing sugars (products of enzyme activity) were measured using DNS reagent test since the optical absorbance can be more accurately measured numerically using spectrophotometer at 540nm.

2.6.1 Dinitrosalicylic acid method.

Dinitrosalicylic acid reagent was prepared by adding 1g 3, 5-dinitrosalicylic acid in 50ml of distilled water. 200mg crystalline phenol (optional) and 30g of Sodium potassium tartrate were added to the solution, which turns the solution into yellow colour. To this, 20ml of 2N NaOH was added. This turns the colour of the solution into transparent orange yellow. Finally, the stock was made into 200ml by adding distilled water. Stock was stored at $4^{\circ}C$ in refrigerator, to prevent deterioration [4].

After the incubation, filter paper strips were carefully removed from the tubes using a glass rod. Then 0.5ml of DNS reagent was pipetted into each tube. This terminated all enzymatic reactions occurred in the tube. Then the lids of tubes were tightly closed, and placed in a water bath at $95-100^{\circ}C$ for 10 minutes. After this, tubes were immediately transferred into an ice cold bath and kept for few minutes. 1ml of distilled water was pipetted into each tube before measuring the optical absorbance. Colour change in each tube was measured by using UV spectrophotometer at 540nm wavelength. Finally, the optical absorbance readings were compared and plotted with the standard glucose curve to find the glucose (product) concentrations [4].

From each glucose and buffer mixture, 0.1ml of solution was added to 0.15ml of Trisodium citrate dihydrate buffer solution. Then each centrifuge tube was transferred into a water bath where tubes were incubated at $50^{\circ}C$ temperature for an hour, same as the conditions given for the enzyme filter paper assay. After the incubation, 0.5ml of DNS reagent was pipetted into each tube and the lids of all tubes were tightly closed. Then the temperature in the water bath was raised to $95-100^{\circ}C$ and kept for 10 minutes. Finally, the tubes were immediately transferred into an ice cold bath for few minutes and 1ml of distilled water was pipetted to each tube before measuring the absorbance of optical absorbance, and the samples were examined for the colour change.

Colour change in each tube including the control blank was measured by using UV spectrophotometer at 540nm wavelength. Finally, the optical absorbance readings were plotted against the concentration of glucose.

Table 2: Glucose concentration vs. Optical absorption at 540nm

Glucose concentration	Optical absorption
3.35mg/0.5ml	0.766
2.50mg/0.5ml	0.580
1.65mg/0.5ml	0.378
1.00mg/0.5ml	0.228

As given in the table 2 optical absorbance differ according to the concentration of glucose, this is ranging from 1.00mg/0.5ml to 3.35mg/0.5ml resulted in optical absorbance ranging from 0.228 to 0.766 respectively.

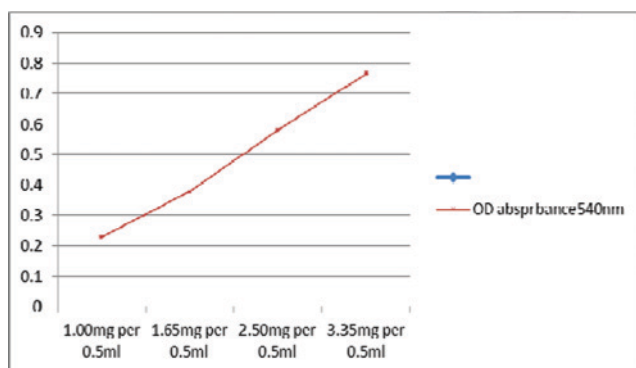


Figure 2: Glucose concentrations vs. Optical absorbance (540nm): - standard glucose plot

This standard curve (Figure 2) was used to find the unknown concentrations of reducing sugars in all samples, dilutions used were translated into enzyme concentrations. Concentration of enzyme which would have released exactly 2.0 mg/ 0.5ml of glucose by means of a plot of glucose liberated against the logarithm of enzyme concentration was estimated.

Filter paper unit was calculated according to IUPAC-FPU. As given below:

$$FPU = \frac{0.37}{\text{Enzyme concentration to release 2mg glucose}} \text{ units} \cdot \text{ml}^{-1}$$

This quantitatively shows the activity of cellulolytic enzymes.

Percentage of saccharification of cotton wool by each enzyme is calculated by using given formula.

$$\text{Saccharification}(\%) = \left\{ \frac{\text{Glucose (mg/0.5 ml)}}{\text{Substrate (mg/0.5 ml)}} \right\} \times 100$$

Since cotton wool consists of 94% cellulose, substrate concentration in 0.5ml can be derived as 47mg (cellulose). By applying the product (glucose) concentration retrieved from the standard glucose curve the percentage of saccharification was calculated.

2.7 Identification of fungi

In order to identify the fungal colonies colony colour, shape, border, and spots (if the spores are available) were recorded as given in table 4.2. Microscopic visuals were observed under high power (40×10) oil immersion objective. Spores and the mycelia were observed so clearly (Figure 3), and the data were recorded and used in classification.

Fungi were classified up to the genus level by their morphological features. Classification was based on microscopic observation of mycelia as well as reproductive structures such as spores and fruiting bodies, if available. Characters used in classification were compared by considering mycelial characters such as presence of septa, whether mycelium was branched or not, on mature colonies the presence of reproductive structures such as sporangia, conidia and their morphology, types of spore they generate, whether spores are septate or not and position of rhizoids on the mycelium etc.

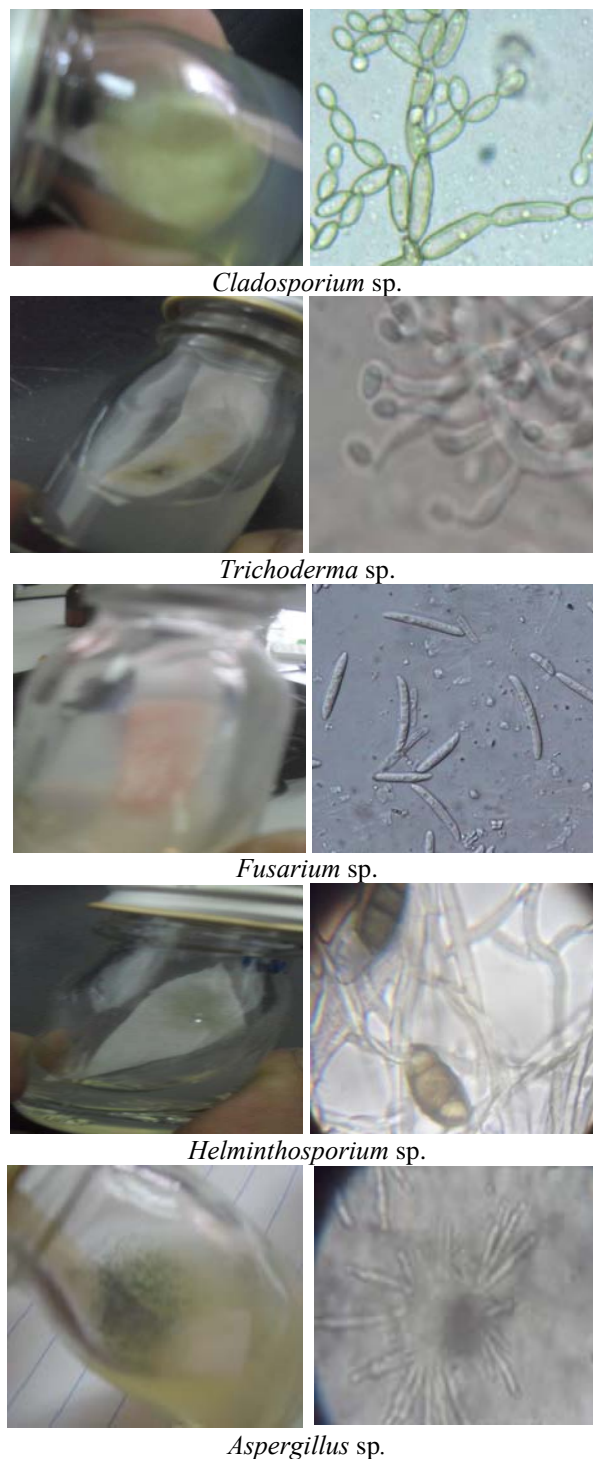


Figure 3: Fungal samples

2.8 Effects of pH and Temperature on fungal enzyme activity

Enzyme activity related to variation in temperature and pH were measured separately. Filter paper assays of each fungal crude enzyme were kept in water baths at temperatures of 37^o, 50^o and 60^oC. After an hour of incubation DNS reagent test was done.

Similarly, pH of the each buffer solution was changed to 3, 6, 8, and 13 by adding either dill NaOH or dill HCl. Then crude enzyme of each fungus was added and filter paper strips were placed. Assay was incubated at 50^oC for an hour and products were measured using DNS reagent test. Concentrations of reducing sugars were obtained from the standard glucose curve and, finally activity of enzymes was calculated as FPU/ml.

3. Results and discussion

3.1 Fungal colony on cotton wool broth

After 4 days of incubation at room temperature (25^oC) partially saccharified cotton wool in CBM (cellulose basal medium) containing cream colour colony of *Trichoderma* sp. (Figure 3). Colony of *Trichoderma* sp. is cream colour at the early stage later becomes green due to abundant sporulation.



Figure 3: Fungal colony on cotton wool immersed in CBM medium after 4 days of saccharification at 25^oC

3.2 Saccharification of cotton by enzymes of fungi

According to the results (Figure 4) *Trichoderma* sp. shows the highest saccharification of 1.25%, which is immediately seconded by the *Aspergillus* sp. of 1.23%, while *Helminthosporium* sp. and *Cladosporium* sp. gives moderate saccharification of 1.00 and 0.97% respectively.

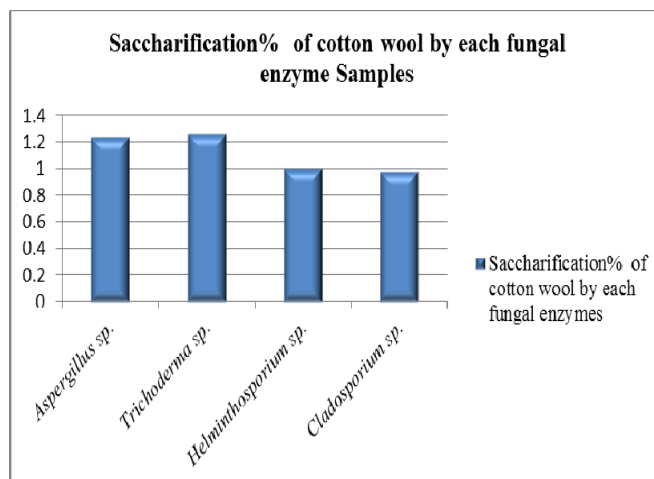


Figure 4: Saccharification% of cotton wool by each fungal enzymes

3.3 Activity of fungal enzyme from cotton wool broth (by filter paper assay).

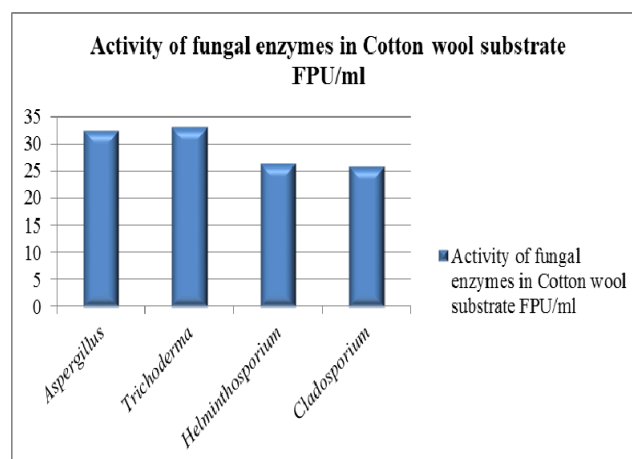


Figure 5: Activity of fungal enzymes from cotton wool substrate in cellulose basal medium

Enzyme activity is determined by the binding ability of their enzymes to the available substrate. According to the graph (Figure 5) *Aspergillus* sp. and *Trichoderma* sp. respectively showed high activity of 32.52 and 33.08 FPU/ml, while *Helminthosporium* sp., *Cladosporium* sp. depicted relatively low activity of 26.35 and 25.78 FPU/ml.

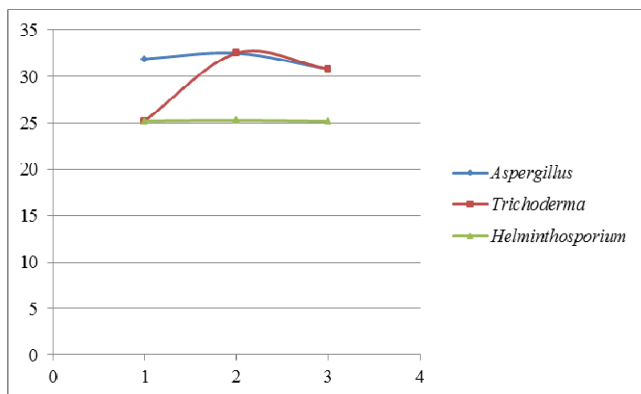
3.4 Effect of environmental factors on saccharification of cotton. (Based on examining derived enzyme activity by filter paper assay)

Environmental parameters are one of the major factors in saccharification of fungal enzymes. Mainly during the storage of cotton humidity, temperature, and even pH of the substances come to contact may facilitate colonization of fungi. However in case of temperature, there is no proved relationship between temperature of colonization and optimal temperature of their enzymes. In addition preference of such parameters by each fungal species may differ.

3.4.1 Effect of temperature on the activity of cellulolytic fungal enzymes (FPU/ml).

The rate of an enzyme catalyzed reaction increases as the temperature has risen. Variations in reaction temperature by

1 or 2 degrees may introduce changes of 10 to 20% in the results. In this experiment, enzymatic reaction of given fungi (Figure 5) showed a peak at 50°C by reaching a peak of 32.5 FPU/ml. This shows that the temperature for the cellulolytic enzymes of three given fungi was 50°C. However, if further high temperatures are tested it is possible to find the point they get denature. Normally animal enzymes get denatured even at 40°C. Nevertheless, for fungi it is higher. It is also possible to observe the tolerance as well as the preference of high temperature (since 50°C as optimum) by fungal enzymes. According to the graph, the fungus *Trichoderma* sp. shows an increase in the rate of reaction until 50°C followed by decline afterwards.

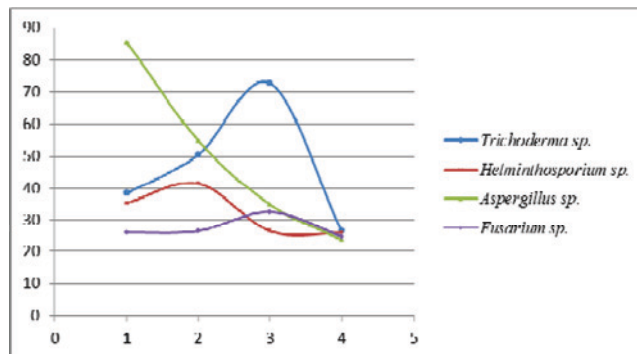


X axis: 1: 37°C, 2: 50°C, 3: 60°C Y axis: FPU/ ml

Figure 5: Temperature vs. activity of fungal enzymes (FPU/ml).

3.4.2 Effect of pH on the activity of cellulolytic fungal enzymes (FPU/ml)

Since enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has its own optimum range for pH, where it is most active, and the result is determined by the effect of pH on a combination of factors such as binding of the enzyme to substrate, catalytic activity of the enzyme, ionization of the substrate, and the variation of protein structure. The initial rates for many enzymatic reactions exhibit bell-shaped curves. The most favourable pH value (optimum pH) may vary among enzymes of different fungi. In this experiment (Figure 6) the optimum pH for fungus *Trichoderma* sp. and *Fusarium* sp. was closer to neutral, and for *Helminthosporium* sp. it is 3. It means *Helminthosporium* sp. prefers slightly acidic medium. For *Aspergillus* sp. the curve was peaking at very low pH that showed the preference of *Aspergillus* sp. towards acidic environment.



In X axis: 1:pH3, 2:pH6, 3:pH8, 4:pH13 Y axis: FPU/ml

Figure 6: Effect of pH on the activity of cellulolytic enzymes from fungi.

4. Conclusion

Based on empirical calculations *Trichoderma* sp. and *Aspergillus* sp. depicts high cellulolytic activity of 33.08 and 32.52 FPU/ml respectively on raw cotton, in addition their saccharifying ability depicted 1.25 and 1.23%. This is considerably higher when compare to other varieties. *Trichoderma* sp. is a soft wood rotting fungi which is found in moist soil [6]. In a similar work, about 55-66% of cotton saccharifying activity was recorded by the culture containing a mixture of both *Trichoderma* sp. and *Aspergillus* sp [5]. Since fungal spores are airborne their spreading is rapid and cannot be controlled by any means unless keeping the cotton away from dust particles. High substrate concentration (94% of cellulose) and hygroscopic nature makes cotton as extremely susceptible to any fungal colonization. Further, this study also paves way to experimental aspects of enzyme kinetics, the optimal pH and the favourable temperature for each fungal enzyme activity during saccharification was affirmed.

5. Future Scope of this study

Application of extracted cellulolytic fungal enzymes in cotton waste management is the main concern here, this may leads to waste reduction from textile mills and production of biofuel (ethanol) by using enzyme treated cotton in fermentation. In addition there is a room to study about the saccharified cotton pulp in a useful manner. However, this work also provides some raw facts towards the protection of cotton garment from fungal attack this can be further evaluated for the production of synthetic resin or any other coatings with anti-fungal properties.

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