Study of morphological changes and natural degradation in agarwood (*Acquilaria agallocha* Roxb.) bark-based Sanchi manuscripts of Assam, North East India

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No experimental study has been done so far on the natural degradation of the traditional Sanchi manuscripts made from the bark of agarwood (aloeswood, i.e. *Acquilaria agallocha* Roxb., locally known as Sanchi tree in Assam, North East India). In this study, SEM, FTIR, XRD and stress–strain test using UTM were carried out on lignocellulosic Sanchi manuscripts to determine cellulosic variations. Reduction of crystallinity index of cellulose, decrease in tensile strength and toughness indicated the natural degradation of Sanchi manuscript. The study reveals that the Sanchi manuscripts are highly vulnerable to natural degradation, and therefore need scientific techniques and treatments to prolong lignocellulosic changes.

Keywords: Agarwood, cellulose crystallinity, lignocellulosic degradation, morphological changes, traditional manuscripts.

THERE have been worldwide concerns about conserving wood-based manuscripts and other similar cultural legacies. These are most vulnerable to deterioration due to temperature, humidity, light, fungal attack and other environmental factors. Most of the ancient manuscripts of Assam, North East India, were written on the bark of agarwood (*Acquilaria agallocha* Roxb.), which was seasoned and treated with *Phaseolus radiates*, yellow orpiment (arsenic sulphide; As_2S_3) and vermilion (mercuric sulphide; HgS) for colouring and protection. This practice dates back to King Pushyavarman of the 4th century AD, the first king of the Varman Dynasty of Kamarupa (ancient Assam). However, according to a report of the National Mission for Manuscripts (2005–06), almost 42,000 wood-based manuscripts still exist in Assam, and with time, these are degrading due to environmental factors and biological agents. Assam being a subtropical region with high average temperature and relative humidity (RH), the problem of biodeterioration is prominent. As reported by Agrawal and Barkeshli[1], the optimum environmental conditions for the growth of fungi are temperatures in the range of 20°–30°C and RH above 65%. Various studies have reported that the change in cellulosic composition is caused by microbial growth[2,3]. As Sanchi manuscripts are wood-based lignocellulosic, they are naturally composed of mainly hemicellulose, cellulose and lignin, and hence, susceptible to enzymatic and chemical reactions such as oxidation, hydrolysis and dehydration reactions[4]. During the biodegradation process in wood-based manuscripts, cellulose is changed into a glucose molecule by cellulolytic fungi possessing a system of extracellular and intracellular enzymes known as cellulase[5]. Cellulase hydrolyses the hemicelluloses (carbohydrate polymers) in the cell wall into digestible units and weakens crystalline cellulose, which is primarily responsible for the strength of the cell wall[6].

Studies on the cellulosic content of wood have popularly used laboratory experiments like scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR). SEM is a quick and reliable method for identifying microbial growth, providing a three-dimensional microstructure of cellulose fibre at a high magnification of 500–100,000×. It helps identify the morphological changes induced by fungal growth[7]. FTIR analysis of wood-based samples quickly identifies the structural and chemical changes induced by common fungal degradation[8]. It also measures the structural transformation of lignin and cellulosic components[9,10]. XRD detects the interference pattern generated when X-rays encounter the regularly spaced crystalline cellulose planes in wood-based manuscripts, which has an important bearing on the physical, mechanical and chemical properties of cellulose fibre, viz. Young's modulus, dimensional stability, density and hardness[11]. The wood-decaying fungi reduce the strength of these manuscripts by metabolizing the crystalline cellulose fraction. Therefore, this study aimed to understand the physical or morphological degradation of Sanchi manuscripts with time caused by fungal infestation and the resultant changes in cellulose composition of these manuscripts studied using SEM, FTIR and XRD.

Material and methods

To determine the properties of Sanchi materials belonging to different time periods, five samples were considered for

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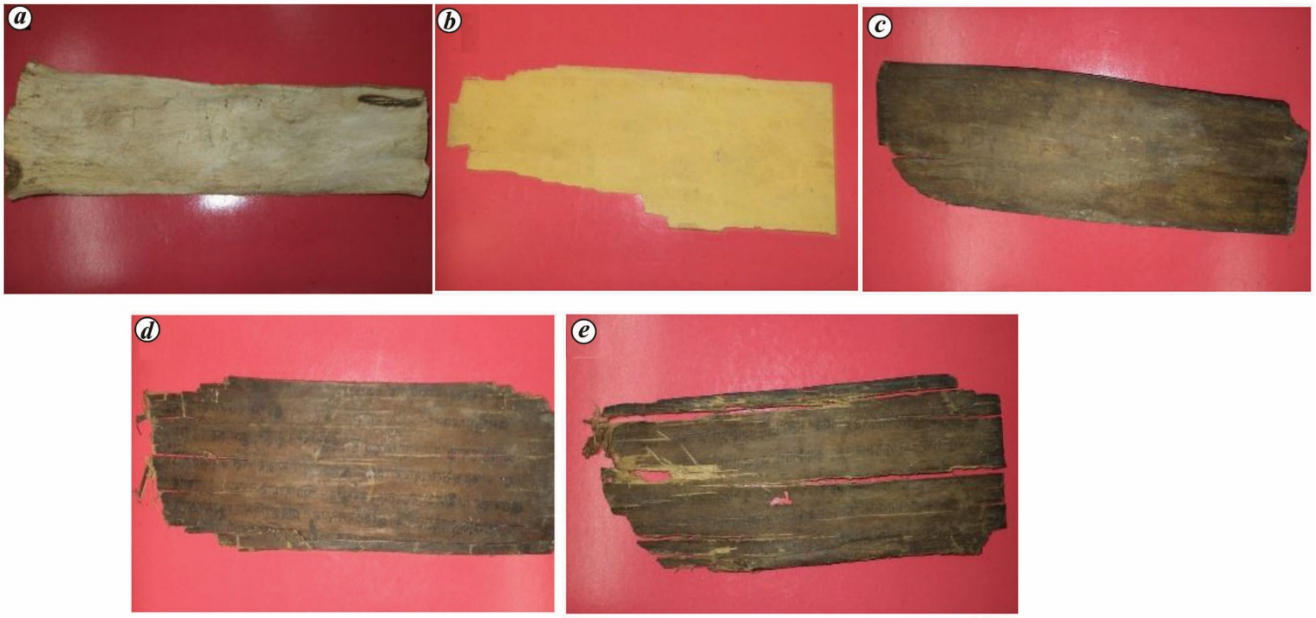

Figure 1. Sanchi manuscript samples of various time-periods considered in this study. ***a***, Fresh Sanchi manuscript (sample 1). ***b***, Hundred year old Sanchi manuscript (sample 2). ***c***, Two hundred year old Sanchi manuscript (sample 3). ***d***, Three hundred year old Sanchi manuscript (sample 4). ***e***, Four hundred year old Sanchi manuscript (sample 5).

Table 1. Characteristics of Sanchi manuscripts

Sample	Characteristic	Size (cm × cm)
1	Freshly processed Sanchi manuscript	21 × 6
2	Processed, unused, Sanchi manuscript blank folio of about 100 years old	21 × 6
3	Processed, unused, Sanchi manuscript blank folio of about 200 years old	21 × 6
4	Processed Sanchi manuscript folio of about 300 years old	21 × 6
5	Processed Sanchi manuscript folio of about 400 years old	21 × 6

this study (Figure 1 and Table 1). The age of the samples has been determined on the basis of the colophon, a written statement provided at the beginning of the manuscript text showing the specific date and time of its preparation in literary form, and also on the basis of the verbal communication made with the owner of the manuscripts.

SEM analysis

A scanning electron microscope (Leo 1430vp) was used with an acceleration voltage of 10 kV and electron beam capacity of 300 mA. A 5 mm square piece of each sample (1–5) was attached to the metal stub in the microscope one at a time. To improve the conductivity of the samples and the quality of the SEM images, the samples were coated with a thin layer (200 Å) of gold–palladium alloy using scanning electron microscope (SEM BALTEC MED 020).

X-ray diffraction

XRD analysis of five pulverized samples was performed (Bruker D2 PHASER diffractometer). The powdered specimens were pressed into a 15 mm × 20 mm tablet to a thickness of 1 mm. The specimens were measured in the 2θ range between 10° and 80°. The set-up was operated at a voltage of 40 kV and current density of 30 mA with the diffracted intensity of Cu Kα radiation ($\lambda = 0.1542$ nm) and scan speed of 0.05°/s.

The cellulose crystallinity index was calculated as follows

$$\text{CrI}\ (\%) = (I_{002} - I_{\text{AM}})/I_{002} \times 100,$$

where I_{002} is the maximum intensity of the 002 diffraction peak and I_{AM} is the minimum intensity of the peak representing amorphous intensity.

FTIR analysis

Two milligram each of powdered manuscript samples (1–5) and dried KBr (350 mg) were placed in an agate mortar, mixed properly and pulverized to obtain a uniform composition. The mixture was dried at 60°C for 4 h and then poured into a tabletting mould to form transparent tablets. The spectra were recorded using a Fourier transform infrared spectrophotometer (Nicolet IS-10) set to a resolution of

4 cm^{-1} over the range 4000–400 cm^{-1} with 100 scans per sample.

Stress–strain tests

To assess the deformation of the Sanchi manuscripts, specimens (1–5) of 150 mm × 50 mm were subjected to axial loading using a Universal Testing Machine (Instron). For accuracy of the result, the specimens were placed at the centre of the crosshead with their end perpendicular to the longitudinal axis. To ensure adequate grip, a length of 50 mm was left at both ends for clamping the specimens. The experiments were conducted at a constant crosshead speed of 2 mm/min.

Chemical treatment using EDTA disodium and alkaline hydrogen peroxide with silicon dioxide

EDTA disodium and alkaline hydrogen peroxide were treated for strengthening of the damaged cellulose and remove stains from the samples. First, the Sanchi manuscript samples were treated with 5% EDTA disodium solution for 1 min (Figure 2). Next, they were treated with a solution of 30% alkaline hydrogen peroxide and silicon dioxide for 1 min (Figure 3) and left for 24 h untouched under natural conditions for observation.

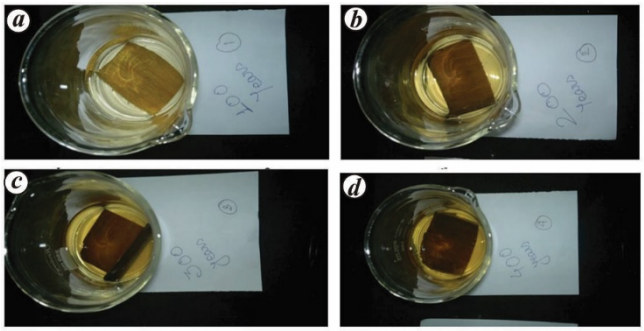

Figure 2. Infested Sanchi manuscripts of different time periods tested with 5% EDTA disodium solution. ***a***, Hundred year old sample. ***b***, Two hundred year old sample. ***c***, Three hundred year old sample. ***d***, Four hundred year old sample.

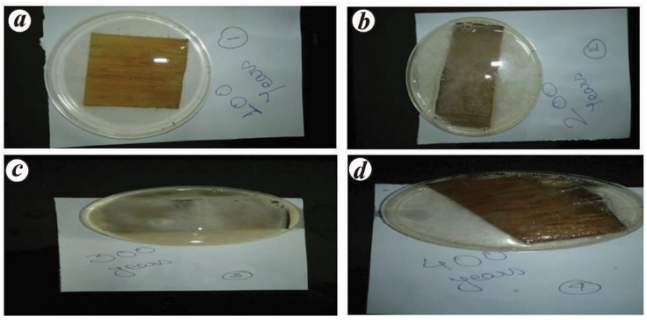

Figure 3. Infested Sanchi manuscripts of different time periods tested with alkaline hydrogen peroxide and silicon dioxide. ***a***, Hundred year old sample. ***b***, Two hundred year old sample. ***c***, Three hundred year old sample. ***d***, Four hundred year old sample.

Results and discussion

SEM analysis

SEM analysis indicated surface modifications induced due to morphological changes in the samples (Figures 4 and 5). The fresh sample showed the least degradation, while the 400-year-old sample showed maximum degradation caused to the cellulose fibres in the form of a gradual depletion with the age of the sample and the nature of the fungal infestation. Due to various metabolic activities and enzyme secretion by the fungal colonies, the infected manuscripts showed various coloured patches on their surfaces, especially samples 3–5, recorded before SEM analysis. Colonies made by different types of fungi and their secretions result in different stains of visible colours. The visual assessment and analysis of these stains confirm possible fungal infestation. In the present study, the manuscript samples exhibited white, green, black, deep brown and light yellowish stains indicative of fungal infestation by *Aspergillus*, *Chaetomium* and *Penicillium,* which attack and deteriorate cellulose[7,12]. In addition to the degradation of cellulose, there is decomposition of lignin, which acts as a binder and filler in all cellulosic materials.

FTIR analysis

Figure 6 shows the FTIR spectra obtained from the samples. The spectra exhibit a strong O–H stretching at 3300–3400 cm^{-1}, C–H stretching at 2800–3000 cm^{-1} and several distinct peaks in the fingerprint region between 500 and 1750 cm^{-1}. Most bands have contributions from carbohydrates (cellulose and hemicellulose) and lignin. Microbial degradation results in a reduction of hemicellulose content and, therefore, an increase in the relative amount of lignin[13].

In order to determine the rate of lignin decay and carbonyl formation, the intensities of the carbonyl absorption band at 1100 cm^{-1}, lignin reference band at 1670 cm^{-1} and carbohydrate reference bands at 1372 cm^{-1} were measured, and their ratios determined (Table 2). The lignin/carbohydrate ratio (I_{1670}/I_{1372}) from samples 1, 2, 3, 4 and 5 was found to be 1.67, 1.46, 1.23, 1.10 and 0.91 respectively. The carbonyl/carbohydrate ratio (I_{1100}/I_{1372}) was 1.37, 1.26, 1.21, 1.09 and 0.96 respectively.

Popescu *et al.*[14] have reported similar findings for lime wood in which the ratio between the absorption intensities of the band at 1280 cm^{-1} (assigned to the C–H bending mode) and the band at 1200 cm^{-1} (assigned to the C–O–C stretching mode of the pyranose ring) (I_{1280}/I_{1200}) was used to determine the degree of crystallinity for the samples. They found a decreasing trend of crystallinity from 0 to 84 days for the samples. Dobrica *et al.*[15] reported similar results

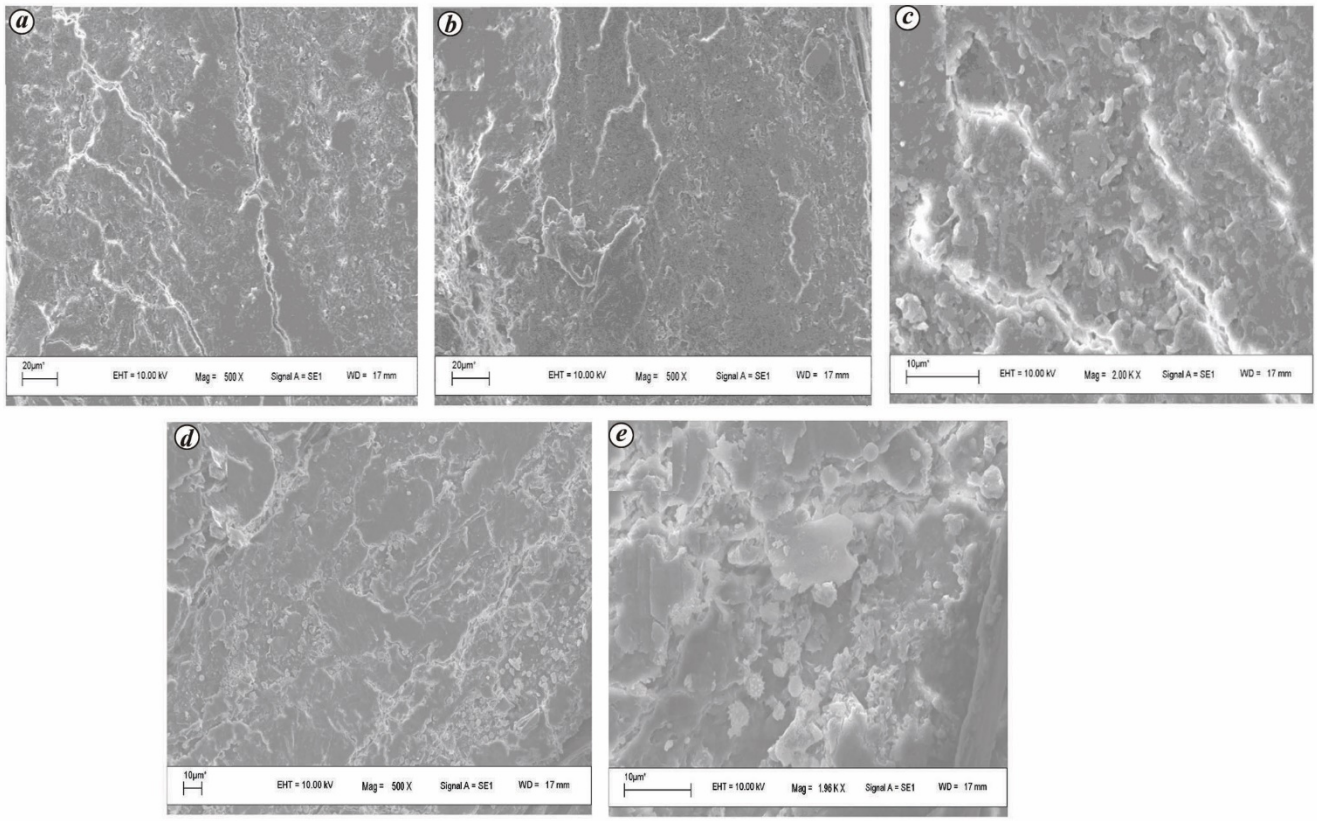

Figure 4. SEM images showing morphological changes and fungal infestation on the flat top surface of five Sanchi manuscripts samples of different time periods. ***a***, Fresh sample (sample 1). ***b***, Hundred year old sample (sample 2). ***c***, Two hundred year old sample (sample 3). ***d***, Three hundred year old sample (sample 4). ***e***, Four hundred year old sample (sample 5).

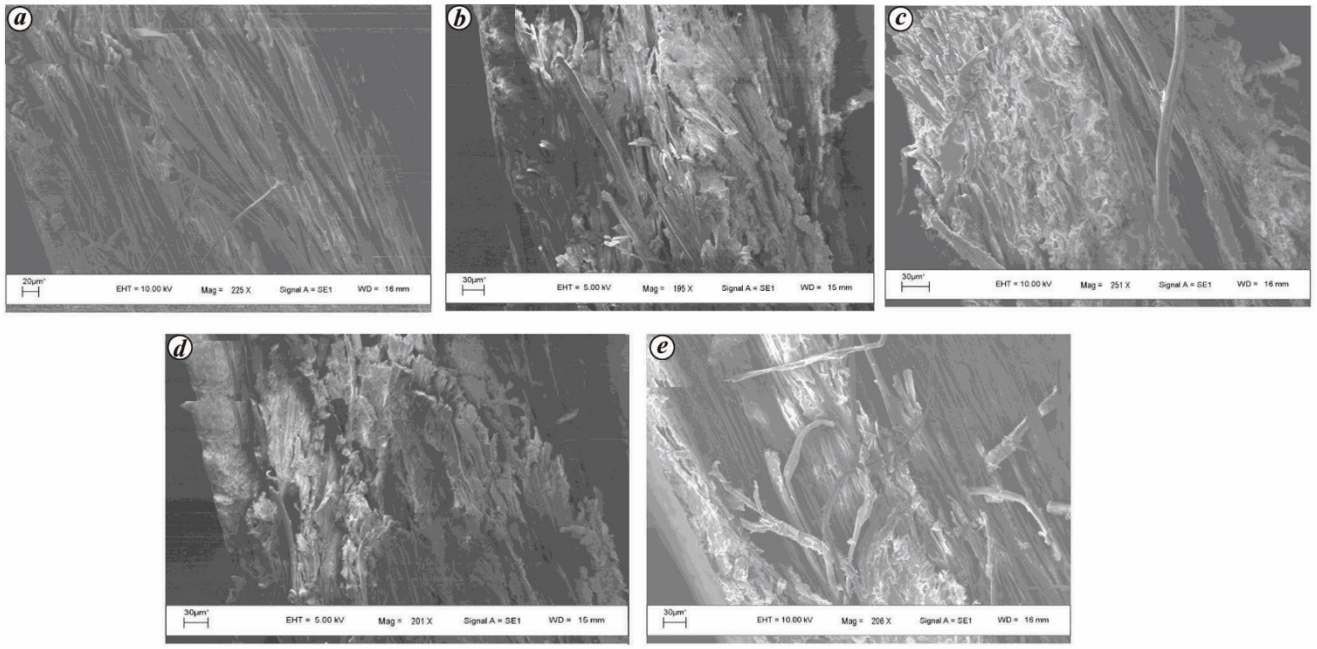

Figure 5. SEM images showing edge surface morphology of five Sanchi manuscript samples of different time periods. ***a***, Fresh sample (sample 1). ***b***, Hundred years old sample (sample 2). ***c***, Two hundred year old sample (sample 3). ***d***, Three hundred year old sample (sample 4). ***e***, Four hundred year old sample (sample 5).

Table 2. Changes in lignin/carbohydrate and carbonyl/carbohydrate peaks

Sample	Carbonyl reference band intensities at 1100 cm^{-1}	Carbohydrate reference band intensities at 1372 cm^{-1}	Lignin reference band intensities at 1670 cm^{-1}	Lignin/carbohydrate ratio I_{1670}/I_{1372}	Carbonyl/carbohydrate ratio I_{1100}/I_{1372}
1	57.02	41.52	69.34	1.67	1.37
2	54.33	43.09	62.91	1.46	1.26
3	41.96	34.68	42.66	1.23	1.21
4	34.94	31.99	35.19	1.10	1.09
5	28.84	29.92	27.22	0.91	0.96

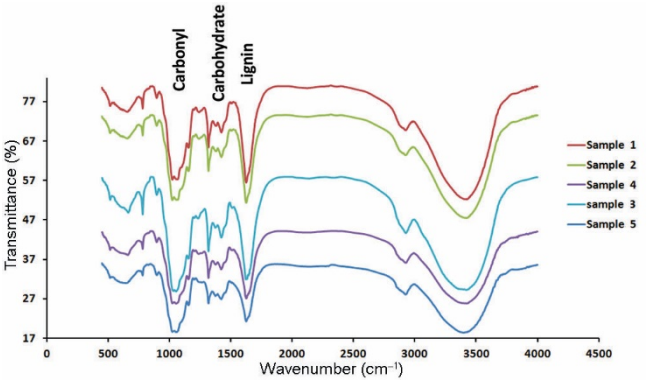

Figure 6. Fourier transform infrared spectral plots.

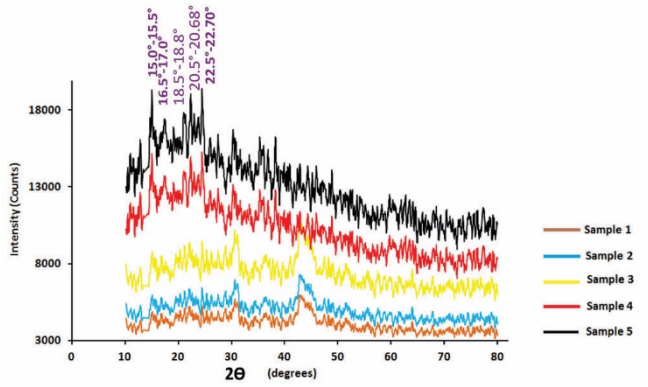

Figure 7. X-ray diffraction spectrum.

for oak, acacia and hornbeam wood. They found that the lignin/carbohydrate ratio and carbonyl/carbohydrate ratio decreased with the period of usage. Pandey and Pitman[8] conducted a study on the effect of white-rot *Phanerochaete chrysosporium* on Scots Pine wood (*Pinus sylvestris* L.) and Beech wood (*Fagus sylvatica* L.) for different durations (2–12 weeks). The calibrated FTIR data revealed that the lignin/carbohydrate ratio gradually decreased as the decay progressed, indicating a decrease in lignin content during a six-week period. The decrease in lignin content indicated an advanced degree of microbial degradation, supporting the findings of other similar studies. Therefore, the gradual decrease of the lignin/carbohydrate ratio and carbonyl/carbohydrate ratio, as found in the FTIR spectra of the Sanchi manuscripts, indicates degradation of its lignocellulosics due to microbial infestation.

XRD analysis

Figure 7 shows the XRD spectra of the five samples. The spectra obtained with 2θ reflection around 15.0°–15.5° and 16.5°–17.0° are assigned to the (101) crystallographic plane, 18.5°–18.8° to the amorphous phases, 20.50°–20.68° to the (102) crystallographic plane and 22.50°–22.70° to the (002) or (200) crystallographic plane of cellulose I.

The XRD spectra show considerable variation at the 002 diffraction peak ($2\theta = 20.002°$), associated with the crystalline region of cellulose in slash pine. To find the crystallinity index, a peak-height method was used considering the ratio of the crystallographic plane at 002 ($2\theta = 22.86°$) having the highest peak and the amorphous plane ($2\theta = 18.80°$) having the flattest peak[16]. Table 3 shows the crystallinity index for samples 1–5 which shows a decreasing trend.

Popescu *et al.*[14] documented similar results, as the crystallinity index gradually decreased from 0 to 84 days for lime wood exposed to the fungus, *Trichoderma viride*. Howell *et al.*[12] reported a similar decrease in the crystallinity index of spruce blocks inoculated with the fungi *Meruliporia incrassate* and *Chaetomium elatum* for four time periods: week 2, week 4, week 6 and week 8. The position of the bands for crystalline cellulose was found to be slightly shifted to higher theta values from samples 1 to 5 in the 002 crystallographic plane (22.30°, 22.45°, 22.57°, 22.75° and 22.88° respectively). The position of the bands for the amorphous zone showed a decrease in theta values for samples 1 to 5 in the amorphous plane (13.50°, 10.60°, 7.40°, 4.30° and 2.70° respectively), indicating a decrease in the *d*-spacing. Popescu *et al.*[14] also reported a decrease in *d*-spacing after the degradation of lime-wood blocks by fungi. The degree of crystallinity was found to decrease as the biodegradation process advanced. Therefore, it can be concluded that fungal infection in wood is the major cause of the decrease in the degree of crystallinity.

Stress–strain test

Figure 8 and Table 4 show the stress–strain test results for the five samples obtained using the Universal Testing Machine.

Figure 7 shows that the stress–strain curves of the samples are almost bilinear up to the ultimate strain. When the

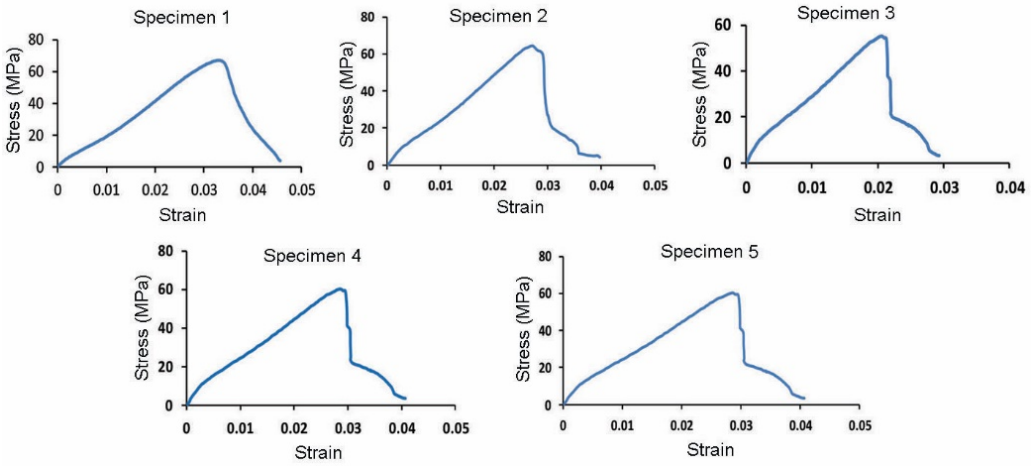

Figure 8. Stress–strain curves of the Sanchi manuscript samples.

Table 3. Crystallinity index of the Sanchi manuscript samples

Sample	Diffraction peak at crystallographic plane ($I_{22.6}$)	Degree of crystallinity at crystallographic plane (Cr (002))	Diffraction peak at amorphous plane ($I_{18.8}$)	Degree of crystallinity at amorphous plane (Cr (am))	Crystallinity index (CrI (%))
1	5,200	22.30	4,500	18.9	13.5
2	6,200	22.45	5,545	18.5	10.6
3	9,500	22.57	8,800	18.4	7.4
4	15,000	22.75	14,350	18.3	4.3
5	19,000	22.80	18,490	18.1	2.7

Table 4. Stress–strain test results

Sample	Young's modulus (MPa)	Tensile strength (MPa)	Toughness (MPa)
1	2,746	66.92	111.40
2	2,688	64.20	87.61
3	2,563	60.18	86.20
4	2,356	55.06	56.96
5	2,128	52.28	53.97

maximum stress occurs, there is matrix failure followed by fibre failure at breaking elongation.

The tensile strength of samples 1 to 5 was 66.92, 64.20, 60.18, 55.06 and 52.28 MPa respectively, showing a gradual decrease from sample, thus exemplifying the fact that the capacity to withstand stress decreases with a decrease in matrix strength with time. Young's modulus showed a decreasing trend with time for samples 1 to 5, i.e. 2746, 2688, 2563, 2356 and 2128 MPa respectively. The toughness of the specimens also showed a decreasing trend for samples 1 to 5, i.e. 111.40, 87.61, 86.20, 56.96 and 53.97 MPa respectively, indicating a loss in the gradual energy absorption capacity and thus increasing the possibility of breakage with time. This range of Young's modulus and tensile strength for Agarwood (Sanchi manuscripts) was similar to those reported in other studies[17,18], and on different types of hardwood.

EDTA disodium and alkaline hydrogen peroxide with silicon dioxide treatment

EDTA disodium acted as a cleaning agent and dissolved metallic ions deposited as dirt on the surface of the Sanchi manuscript samples. It also softened the brittle part of the samples, thereby consolidating cellulose to a substantial extent. The samples were found to regain strength, and some of their original properties were reported. Alkaline hydrogen peroxide and silicon dioxide solution acted as a bleaching agent to remove dark stains of pigmentation caused by metabolites, which were non-reactive to EDTA disodium, without damaging the cellulose content of the manuscript samples.

Conclusion

SEM analysis clearly showed the surface morphology of the samples, indicating gradual deterioration due to physical causes and fungal infection.

With the help of XRD tests, the crystallinity index was calculated from the height ratio between the intensity of the crystalline portions and the total intensity of the samples. The degree of crystallinity was found to decrease as the biodegradation process advanced. Therefore, fungal infection of the manuscripts is the major cause of the decrease in the degree of crystallinity.

With time, there has been a degradation of the lignocellulosic fabric, which is indicated by increasing CO absorption as observed in the infrared spectroscopy, i.e. with the formation of the –COOH group and increased acidity of the specimen, which could be due to aerial oxidation that makes the specimen more fragile. The results from SEM analysis, stress–strain test and XRD and FTIR studies are consistent.

The stress–strain tests using UTM showed a gradual decrease with Young's modulus, indicating that the capacity to withstand stress decreases from the fresh to 400-yr-old sample with a decrease in the strength of cellulose fibres due to various natural causes and fungus infestation.

Chemical treatment with EDTA disodium, alkaline hydrogen peroxide and silicon dioxide solution showed good results, which may help in the development of a scientific and effective process of restoration of microbial-infested Sanchi manuscripts, and also help preserve them for a longer period of time in the future.

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