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Investigation of Microorganisms Deteriorating Ancient Ola Leaf Manuscripts

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Abstract: Ola leaf manuscripts from Sri Lanka date back to several centuries. While they have been well preserved over the last century, their condition has worsened in recent years when black dots caused by microorganisms started occurring on their surface. In this study, the current state of preservation and the factors causing deterioration are examined using microscopy techniques. Microscopic images clearly show that the manuscripts are contaminated by microorganisms which penetrated deeply into the carrier material, destroying the internal structure. A *Penicillium griseofulvum* strain was recognized as the most active microorganism in xylan degradation. Sri Lanka's climate provides favorable conditions for the growth of these fungi. Therefore, it is suggested that temperature and humidity of the archival space should be better

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controlled in order to ensure the Ola leaf manuscripts' long-term preservation.

Keywords: xylanase activity, microorganism, Ola leaf manuscripts, Sri Lanka

Zerstörung von Palmblattmanuskripten durch Mikroorganismen

Zusammenfassung: Die Herstellung von Palmblattmanuskripten hat in Sri Lanka eine lange Tradition. Während der Erhaltungszustand dieser Manuskripte in den vergangenen Jahrhunderten stabil war, kam es in den letzten Jahrzehnten vermehrt zu einem Abbau des Trägermaterials durch Mikroorganismen. Diese erscheinen als schwarze Punkte auf der Oberfläche. In der vorliegenden Studie werden der aktuelle Erhaltungszustand und die Schadensfaktoren genauer untersucht. Im Mikroskop ist deutlich sichtbar, dass die Manuskripte von Mikroorganismen kontaminiert sind, die tief in das Trägermaterial eindringen und dessen innere Struktur zerstören. Ein *Penicillium griseofulvum*-Stamm ist besonders am Abbau von Xylan beteiligt. Das Klima in Sri Lanka bietet günstige Bedingungen für das Wachstum dieser Schimmelpilze. Um die langfristige Erhaltung von Palmblattmanuskripten zu gewährleisten wird daher empfohlen, das Klima in Depots und Ausstellungsräumlichkeiten besser zu kontrollieren.

Etude des micro-organismes dégradant des feuilles de manuscrits anciens Ola

Résumé: Les feuilles de manuscrits Ola sont des objets séculaires du Sri Lanka. Alors qu'ils ont bien été préservés au siècle dernier, leur état au 21ème siècle a empiré et on a observé l'apparition de points noirs en surface causés par des micro-organismes. Dans cette étude, l'état actuel de préservation et les facteurs de dégradation sont examinés au moyen de techniques microscopiques. Les images sous microscope montrent clairement que les manuscrits sont contaminés par des micro-organismes ayant pénétré profondément dans le substrat et ayant détruit la structure interne. Une souche de *Penicillium griseofulvum* a été identifiée comme étant le micro-organisme le plus actif de la dégradation. Le climat du Sri Lanka offre des conditions favorables au développement de ces champignons. C'est pourquoi, le contrôle de la température et de l'humidité de l'espace d'archivage devraient être mieux contrôlés afin d'assurer la préservation à long terme.

1 Introduction

Before paper was invented, humans had used clay, stone, and metals to engrave their records. In later times, these materials were replaced by the leaves of trees, bark, and animal skins. Palm leaves were one of the most common materials used in South Asia and Southeast Asia including India, Nepal, Sri Lanka, Burma, Thailand, Indonesia, and Cambodia (Agrawal 1984). Palm leaf is a generic term, and each country or region has a distinctive name for this writing support. In Sri Lanka, palm leaf is known as Ola, in Burma as Lontar, in Thailand as Larn, and in various parts of India as Tula, Sritola or Karalika. Palm leaf manuscripts are commonly found in museums and libraries everywhere in these countries. Palm leaves of *Borassus flabellifer* L. and *Corypha umbraculifer* L. have been most commonly used for making manuscripts in these regions. They are resistant to the attack of insects and are impervious to moisture (Suryanwanshi, Sinha, and Agrawal 1994). In Sri Lanka, leaves of *Corypha umbraculifera* L., called Ola leaf, have been used for writing. *Corypha* leaves must be taken from the plant at a semi-mature condition. The best time of Ola leaves harvesting are the four months following the emergence of young leaves. In the following, traditional manufacturing methods and processes of Ola leaf manuscript preparation will be described. Palm leaves are dried in the sun. After the leaflet is stripped from the leaf, they are cut into the required size, rubbed with plant oil, and kept in the shade for approximately three days. Then the leaflets are boiled with rice and kept in the shade for a week. Boiling can also be carried out in water or milk with the juice of fresh turmeric until the leaves attain the expected yellowish color. Again, plant oil is applied. The main advantage of this method is the removal of acidic impurities and the closing of small holes in the leaves. The leaves will become fire resistant and waterproof in these processes, and will last longer (Padmakumar et al. 2003). However, Ola leaf manuscripts are organic material and susceptible to deterioration in nature. The most common deteriorating factors are hot and humid climate conditions, insects, microorganisms, and improper storage. Obviously, fungi and insects rapidly grow at climatic conditions with 70% relative humidity or more and moderate temperatures between 28 and 30 °C, conditions which prevail in Sri Lanka throughout the year (Prasad Cabral, Ravikumar, and Ramanan 2018). Other conservation problems include leaves sticking together, loss of flexibility, holes caused by insects, discoloration, and dust. Cleanliness and maintenance of storage cabinets are recognized as important factors in the long-term preservation of palm leave manuscripts. Also, Sri Lanka's temperature and humidity conditions call for the installation of artificial devices to maintain a constant temperature and humidity throughout the year. As a conventional method for conserving manuscripts,

natural herbs are used because they can keep insects away and because the application of citronella oil, camphor oil, or lemon grass oil on the surface keep the leaves flexible (Suryanwanshi, Nair, and Sinha 1992). Also, faded letters can be restored by applying charcoal mixed with herb oil. The lifespan of palm leaf manuscripts is about 300–350 years. These manuscripts have been copied from one generation to another by hand. If the manuscript gets old or begins to decay, it is transferred on to a new manuscript for the preservation. However, some original manuscripts in the National Library of Sri Lanka are older and their state of preservation is poor (Kumar, Sreekumar, and Athvankar 2009). Thus, scientific research of the current status of the manuscripts is needed.

The National Library in Sri Lanka has numerous collections of Ola leaf manuscripts. The collection is systematically arranged, well maintained, and kept in special filing cabinets. In spite of these efforts, Ola leaf manuscripts are susceptible to deterioration by different factors. Multiple types of deterioration factors are sometimes observed together. The main damages associated with deterioration include cleavage and splitting of the surface layer, stains, discoloration, and damage caused by insects and fungi. The growth of fungi on Ola leaves is not that common though it tends to appear during the rainy season in monsoonal regions. Fungi appear on the surface as greenish black colonies (Cabral and Querner 2017). The development of fungi can be problematic if the leaf gets wet accidentally. Fungi secrete numerous types of enzymes that degrade organic material. Most of the molds produce enzymes related to degradation of plant polysaccharides, such

Figure 1: Ancient Ola Leaf Manuscripts from Sri Lanka.

as cellulose, hemicelluloses and lignin. These enzymes play important roles in converting natural carbon sources of fungi from mainly plant polymers in the cell into small molecules. Hemicellulose is one of main constituent of plant fiber such as papyrus and palm manuscripts. Hemicellulose plays an important structural role in combining with cellulose and lignin. It is first invaded and degraded by microorganisms prior to the decomposition of cellulose (Dekker 1985). Thus, the prevention of hemicelluloses from destruction by microorganisms may be critical to preserving the entire plant-based material. The major component of palm leaf derived hemicellulose is xylan, which is degraded by xylanase (Plackett 2011). Xylanase is an extracellular enzyme produced by microorganisms (Sunna and Antranikian 1997). Microorganisms deteriorating Ola leaf manuscripts have been rarely studied so far. Furthermore, the enzymatic characteristics of microorganisms in Ola leaves are not fully understood. Therefore, this study aims to examine the enzymatic characteristics of microorganisms isolated from the Ola leaf manuscripts in Sri Lanka.

2 Materials and Methods

For this study, four sheets of Ola leaf manuscripts, about 500-year-old Buddhist scriptures, were provided by the National Library and Documentation Services Board in Sri Lanka (Figure 1). Normally, manuscripts measure $4\text{--}6 \times 33\text{--}36$ cm. Except for the brittle edges of the samples, they were relatively intact and showed little color change or microbial contamination. The sample materials for the experiments were obtained from the edge of the manuscripts. Microscopic observation and sampling of microorganisms were performed using a minimum size sample. The surface and cross section of samples were observed using two types of microscopes. One is a video microscope system (iCamscope MVS-24, SOMETECH INC., Seoul, Korea) with magnification of $2.5\times$. The other is a scanning electron microscope (JSM-5900LV, JEOL, Tokyo, Japan) with magnification of $200\times$, $1000\times$, $2000\times$ of the surface and cross section. The elements on the surface of Ola leaf manuscript were investigated using an energy dispersive spectroscopy (JSM-5900LV, JEOL, Tokyo, Japan).

Four bacterial strains and seven fungal strains were isolated. For sequencing these strains, an internal transcribed spacer primer and the Basic Local Alignment Search Tool (BLAST) were utilized. The fungal strains were maintained on solid potato dextrose agar medium. The spore suspension (3×10^6 spores) of fungal strains was added to a 250 mL Erlenmeyer flask containing 50 mL of medium composed of 0.5% oat spelt xylan, 0.1% yeast extract, 0.07% K_2HPO_4 , 0.02% KH_2PO_4 , 0.1% $(\text{NH}_4)_2\text{SO}_4$, and 0.11% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.0). The strains were

Figure 2: Mobile iCamscope Images of the Surface and the Cross Section of Ola Leaf Manuscripts.

cultured on a rotary shaking incubator at 150 rpm and 28 °C. The supernatant was collected as the crude enzyme-containing fraction for the assay of xylanase activity. Then, the crude enzyme of supernatant was precipitated by ammonium sulfate between 30 and 80% saturation. The precipitate was dissolved in 100 mM sodium phosphate buffer (pH 7.0). The xylanase activities were assayed by 1% of oat spelt xylan as substrate in a 100 mM sodium phosphate buffer of pH 7.0. The

Figure 3: Scanning Electron Microscopy (SEM) Images of the Surface (Left) and the Cross Section (Right) of Ola Leaf Manuscripts.

enzyme was incubated for 30 min at 50 °C. The reduction rates of sugars produced as the result of these assays were quantified using the dinitrosalicylic acid method (Miller 1959). Xylose was used as the standard. A unit enzyme activity was defined as the amount of enzyme that produces 1 μmol of reducing sugar per min at 550 nm using model UV-2550 spectrophotometer (Shimadzu, Tokyo, Japan) (Bailey, Biely, and Poutanen 1992). The positive control was made using a highly active xylanase purified from *Thermomyces lanuginosus* (Singh et al. 2000).

3 Results and Discussion

3.1 Conservation Problems of Ola Leaf Manuscripts

Sri Lanka is a tropical island located in the Indian Ocean. Its location is characterized by high temperature and humidity throughout the year. According to investigations for conservation problems of Ola leaf manuscript samples of the National Library collection, fungi stains were a common and major problem identified in the National Library Ola leaf collection. The humid environmental condition throughout the year has accelerated the fungi growth on Ola leaf manuscripts, even though a broad spectrum of fungicides has been applied. Insect attack was the second common issue identified in Ola leaf manuscripts.

Figure 4: High Resolution Images of the Cross Section of Ola Leaf Manuscripts.

Figure 5: Energy Dispersive Spectroscopy (EDS) Spectrum of the Surface of Ola Leaf Manuscripts.

3.2 iCamscope Images of Ola Leaf Manuscripts

Surface and cross section images of samples from the edge and the central part of an Ola leaf manuscript using a mobile video camera, iCamscope MVS-24, were investigated. Figure 2 illustrates irregular black dots caused by microbial contamination on the surface and in the core area of the leaf and shows the abundant growth of an old fungi colony. It is also observed that internal voids increased by cellular destruction (right-bottom). The possibility that black contaminants might originate from inks is low since they are located opposite to the writing. Ola leaf samples with less color change and cracks and with no obvious damage from microorganisms and insects were selected. However, under magnification microbial attacks became visible that were difficult to see with the unaided eye. Therefore, it is necessary to investigate invisible fungal damage, since microscopic fungal damage can occur in all Ola leaf manuscripts.

3.3 Scanning Electron Microscopy Images of Ola Leaf Manuscripts

The microstructure of Ola leaf manuscripts was recorded by scanning electron microscopy (SEM). According to the SEM images of the surface and of the cross section of the manuscript (Figure 3), the surface appears to have lost its original leaf surface structure, and the inner structure observed in the cross section was weakened with cavities filled with microbial hyphae. Therefore, damage caused by fungi lead to brittleness of the material. The microstructure of the cross section was investigated by high resolution SEM image. The results confirm fungal

Table 1: Microorganisms Identified from the Ola Leaf Manuscript in Sri Lanka.

Type	Scientific name		Source
Bacteria		<i>Bacillus aryabhattai</i> <i>Kocuria marina</i> <i>Roseomonas</i> sp. <i>Sphingomonas pseudosanguinis</i>	Ola leaf manuscript
Fungi	Mold	<i>Penicillium griseofulvum</i> <i>Penicillium</i> sp. <i>Phialosimplex chlamydosporus</i> <i>Pleosporales</i> sp.	Ola leaf manuscript
	Wood rot	<i>Hypoxylon</i> sp. <i>Munkovalsaria appendiculata</i> Uncultured fungus clone L042883	

Table 2: Xylanase Activity of Microorganisms Isolated from the Ola Leaf Manuscript.

Type	Scientific name	Xylanase activity (U mL ⁻¹)
Fungi	Positive control (<i>Thermomyces lanuginosu</i>)	2.115
	<i>Pleosporales</i> sp.	0.214
	<i>Penicillium griseofulvum</i>	1.444
	<i>Penicillium</i> sp.	0.926
	<i>Hypoxylon</i> sp.	0.396
	<i>Munkovalsaria appendiculata</i>	1.196
	Uncultured fungus clone L042883	0.926

colonization (hyphae) and microstructure destruction (cell wall degradation) of the manuscript samples (Figure 4). Silica was detected in the energy dispersive spectroscopy (EDS) image of the surface of manuscript (Figure 5). It is speculated that silica might originate from mud or lime used for changing the color to a more brownish hue. Besides these, turmeric juice or copper ions are natural coloring substances that are used to create dyes (Padmakumar et al. 2003).

3.4 Microorganisms Isolated from Ola Leaf Manuscripts

Species of microorganisms were isolated from the surface and interior part of the Ola leaf manuscripts. All the isolates were identified by sequencing using internal transcribed spacer primers and BLAST search. The most commonly recognized species were *Bacillus* and *Penicillium*. The bacterial types identified from the analyses include *Bacillus aryabhatai*, *Kocuria marina*, *Roseomonas* sp. and *Sphingomonas pseudosanguinis*. Mold and wood rot fungi types, such as *Penicillium griseofulvum*, *Penicillium* sp., *Phialosimplex chlamydosporus*, *Pleosporales* sp., *Hypoxylon* sp., and *Munkovalsaria appendiculata* as well as an uncultured fungus clone L042883 strain were also observed. The details of bacterial and fungi type species found in the samples are summarized in Table 1.

3.5 Xylanase Activity of different Fungal Strains

There are many microbial-derived hydrolytic enzymes, and commercially available cellulase and xylanase are derived from fungi such as *Aspergillus niger* or *Trichoderma viride* etc. (Bhardwaj, Kumer, and Verma 2019). These are microorganisms that are frequently observed in the surroundings, and when attached to an organic surface, they cause discoloration and decompose components, degrading physical stability.

In this study, xylanase activity of microorganisms isolated from Ola leaf manuscripts was investigated. The enzyme activity of xylanase was only determined for fungal strains. Among them, *P. chlamydosporus* was not cultured in liquid phase, and the remaining six strains were tested. As a positive control, *T. lanuginosu*, which has excellent hemicellulose degrading ability was used. As a result, the most active strain was *P. griseofulvum*, followed by *Munkovalsaria appendiculata*. Table 2 summarizes the details of xylanase activity of the isolated strains.

Xylanase is the enzyme which degrades the linear polysaccharide β -1,4-xylan into xylose, thus breaking down hemicellulose which is one of the major components of plant cell walls (Li et al. 2000). Xylanase is a factor that plays an important role in microorganisms that break down the main components of plants and use them as nutrients (Christov, Szakacs, and Balakrishman 1999). Therefore, xylanase active fungi pose a serious risk to organic materials.

4 Conclusion

This study examined the microscopic characteristics of microorganisms associated with the deterioration of ancient Ola leaf manuscripts in Sri Lanka based on the analyses of the images from iCamscope and Scanning electron microscopy (SEM). The manuscripts seem to be well preserved, but well-advanced microbial contamination was observed within their structure. The microorganisms identified in the deteriorated manuscripts, including *Bacillus* and *Penicillium* and others, may be closely related to climate factors in Sri Lanka. High temperature and high humidity in monsoonal South Asia seem to provide favorable conditions for the growth of these microorganisms associated with the degradation of historical manuscripts. Therefore, indoor climate conditions in archives will have to be controlled using appropriate technical equipment. Ideal humidity conditions range between 50 and 60%, because fungi grow mostly in high humidity conditions of above 70%, and manuscripts may dry or crack in low humidity conditions of less than 40%. The proper indoor temperature should range between 20 and 24 °C (ASHRAE 2015). Natural antimicrobials could also help to mitigate the deterioration of Ola leaf artifacts. This study clearly showed that the decrease of microbial-induced degradation is important to a successful long-term preservation of cultural heritage. It is expected that this paper contributes to study how inter-annual variations of the monsoonal climatic conditions affect the deterioration of the written heritage in Sri Lanka.

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