How Raman Spectroscopy Can Be Used to Examine the Structural Changes Caused by Certain *Penicillium* Species on Chrome-Tanned Leather

by

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Abstract

Raman spectroscopy was used to elucidate structural changes of wet blue sheep leather due to the growth of fungi belonging to the genus *Penicillium*. After chrome tanning while leathers are still wet, they are prone to be affected by *Penicillium*, resulting in degradation of leather structure. In this study, different fungi species -*Penicillium glabrum*, *Penicillium commune*, *Penicillium echinilatum*, *Penicillium brevicompactum* and *Penicillium chrysogenum*- were inoculated on wet blues and let grown for 14 months. After 14 months, changes on wet blue structure were investigated with Raman spectroscopy. The results showed that chrome-tanned sheep leathers are affected to different degrees by various fungi from the *Penicillium*.

Resumen

Espectroscopía Raman ha sido utilizada para explicar los cambios estructurales del cuero wet blue de oveja debido al crecimiento de hongos pertenecientes al género *Penicillium*. Luego del curtido al cromo, mientras los cueros están todavía húmedos, son propensos a ser afectados por Penicillium, lo que resulta en la degradación de la estructura de cuero. En este estudio, diferentes especies de hongos -*Penicillium glabrum*, *Penicillium commune*, *Penicillium echinilatum*, *Penicillium brevicompactum* and *Penicillium chrysogenum*- fueron inoculados en wet blues y se dejaron crecer durante 14 meses. Después de 14 meses, los cambios en la estructura del wet blue fueron investigados mediante espectroscopía Raman. Los resultados mostraron que los cueros de oveja curtidos al cromo se ven afectados en diversos grados por varios hongos del *Penicillium*.

Introduction

Commonly found in the environment, microorganisms may cause significant problems for certain branches of industry. During and after the production of many industrial products, microorganisms may develop and damage the product, decay it and reduce its quality, and therefore result in diminishing returns. It is estimated that 2-5% of total industrial products in Europe are damaged by micro-organisms.[1] The protein structure in leather is prone to the attack of microorganisms before and after the tanning processes. [2,3]

Currently, the most commonly used tanning material for leather processes is chrome (III) salts.[4] It is believed that aging chrome tanned leathers leads to olation, oxolation and consequently better fixation.[5] However, while these desired reactions occur during the storage period, microorganism activities may also begin to develop on the leather. The oligodynamic property of chrome salts prevents or delays the growth of certain fungi. However, its protection on the leather against certain types of saprophyte structure such as *Trichoderma, Aspergillus, Penicillium etc.* is inadequate.[6,7] Besides, it has been identified that certain commercial fungicides have not provided sufficient protection against certain fungi although they are used at the dosage recommended by the manufacturing companies.[8] A more in depth research on *Aspergillus and Penicillium* species, which are commonly observed at leather factories, should be given higher priority in order to adopt appropriate measures to fight against them. The growth of fungi may result in undesired stains, offensive odor, irregular dye intake, etc. problems on the leather. Some fungal stains are quickly removed by washing, while some may cause permanent discoloration even on finished leather. One of our previous researches showed that six month growth of *Aspergillus niger, Penicillium sp., Trichoderma viride, Alternaria alternata, Cladosporium sp.* on pickled and chrome tanned leather was removed from the skin without leaving any stains. Stain didn't occur on leather even painted in beige.[9] However, this research has been

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conducted with the assumption that the fungal growth on the leather would cause a change in the leather structure even though they don't leave any visible stain.

In this study, species of different *Penicillium* genus have been inoculated in the leather during wet blue stage. At the end of 14 months, the leathers were examined with Raman spectroscopy to determine whether any structural changes were caused by the fungi. Raman techniuqe is particularly sensitive to very small changes in molecular level. Changes in peak positions, shapes, intensities or the appearance or disappearance of peaks in the Raman spectra would indicate changes in the structure of the leather.

Raman spectroscopy has been recognized as a useful analytical tool in many diverse disciplines. However it is a weak optical phenomenon. Approximately one Raman photon is generated for every 10^6 incident photons. For that reason, lasers, high intensity light sources, are required to be able to produce sufficient number of Raman photons to be detected. That is why Raman spectroscopy was not practically available until the development of lasers in 1960s, even though it was first discovered in 1928. In Raman technique, the molecule is excited to a higher virtual energy state by a laser. While the molecule relaxes back to a lower energy state, it emits photons both elastically and inelastically. The elastic scattering phenomenon is termed as Rayleigh scattering, which has the same frequency as the incident laser light. However there is a small amount of light scattered inelastically, which is called Raman scattering. Raman scattered light has lower energy or frequency than the exciting laser photons. The energy difference between the incident laser photons and scattered Raman photons corresponds to vibrational transition energy of the molecule. A Raman spectrum is a plot of the intensity of scattered Raman photons versus the Raman shift in wavenumber, cm^{-1}. [10,11]

MATERIAL AND METHOD

Material

Skin

This research employs three wet-salted raw skins of native breed sheep. No biocidal agents have been used during either the conservation or processing of the raw skins. They are processed according to conventional leather processing recipe until wet blue. In this research, wet blues have been used after being divided into two pieces. Half the leather has been inoculated with each fungus, and one half is left as a control sample.

Fungi

Penicillium commune, Penicillium glabrum, Penicillium echinulatum, Penicillium chrysogenum and *Penicillium brevicompactum* have been employed. All mould species in this study were supplied from Ege University, Department of Biology, Fundamental and Industrial Microbiology Section from Türkiye.

Medium

Malt extract agar (MEA) (Merck) has been used for the production and stocking of test fungi in the microbiological studies.

Method

Leather Processing

The leathers have been brought to wet blue stage in accordance with the classical garment leather processing prescription.

Microbiological Analyses

Each fungus is inoculated into the tubes that contain MEA. Then, they were incubated at 27°C for one week. After a week, 9 ml sterile pure water was added into each tube, and they were vortexed so that equal distribution of fungus micelles and spores are generated on the skin surface. The skins were wrapped and placed in nylon bags separately and have been held at room temperature for 14 months.

Raman Spectrometer

An echelle based PerkinElmer Raman Station 400 operating at 785 nm was used to collect Raman signal dispersed with a 4 cm^{-1} spectral resolution. Spectral range for each spectrum was from 200 to 3100 cm^{-1}. Data analysis was performed using Igor Pro 6.01 software.

Scanning Electron Microscope (SEM)

Hitachi Model TM-1000 Tabletop Microscope was used to obtain SEM images.

RESULTS AND DISCUSSION

As a result of our studies, we have identified that all *Penicillium* that we inoculated on chrome tanned leather have developed. ***P.glabrum*** began to proliferate latest, at the beginning of the fourthfweek of inoculation. ***P.chrysogenum*** and ***P.commune*** started to grow at the start of the first week; ***P.echinulatum*** within the second week and ***P.brevicompactum*** within the third week. A pinky appearance has been identified especially in *P. commune* in week 24. It has been identified that intense green hyphal development has increased very much especially on the skirt sections of the leather, while a dark green color formation has been observed along the back line of *P. echinilatum*. The growth of fungi on the wet blue and SEM images are given Figure 1-10. An apparent increase has been identified in fungus development after week 24. It has been observed that the growth remained stable until month 14. In addition to these visually obtained findings, the results of the studies with Raman spectroscopy are also very interesting.

Figure 1. *Penicillium brevicompactum*

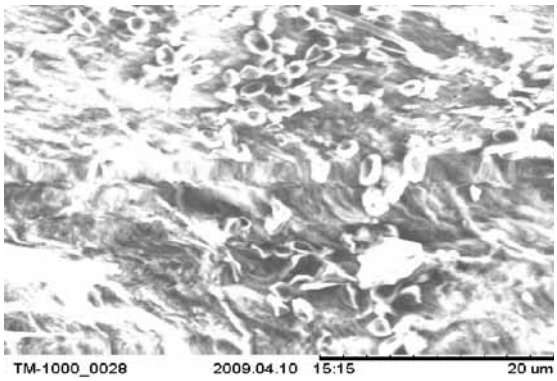

Figure 2. *P. brevicompactum SEM imageX4000*

Figure 3. *Penicillium commune*

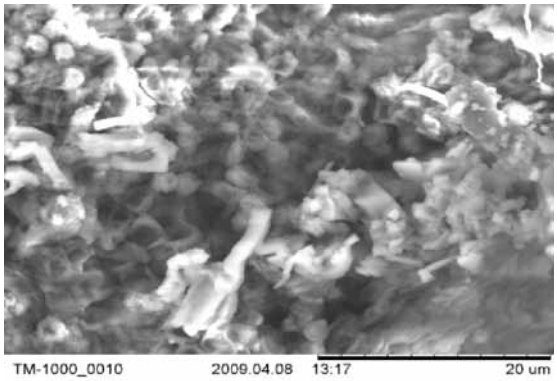

Figure 4. *Penicillium communeX4000*

Figure 5. *Penicillium echinulatum*

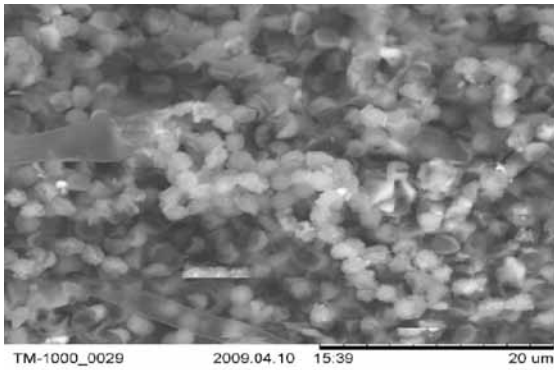

Figure 6. *Penicillium echinulatumX4000*

Figure 7. *Penicillium chrysogenum*

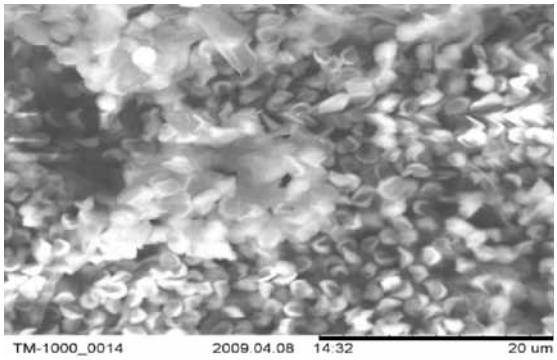

Figure 8. *Penicillium chrysogenum X4000*

Figure 9. *Penicillium glabrum*

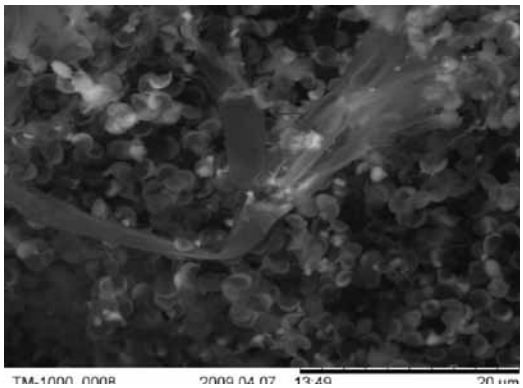

Figure 10. *Penicillium glabrumX4000*

The Raman spectra of wet blue sheep leather as a control sample and those inoculated with *P. glabrum, P. commune, P. echinilatum, P. brevicompactum* and *P. chrysogenum* are shown in Figure 11. Biomaterials intrinsically tend to emit intense fluorescence photons, which cause a broad baseline and make Raman photon detection difficult or in some cases may even completely mask the Raman signal. In order to be able to evaluate spectral differences effectively this broad fluorescence background should be removed and in addition all Raman signal should be brought to the same scale by normalizing the spectra. We applied an iterative baseline subtraction procedure with polynomial fitting[12] to remove the background. Then, each spectrum is normalized to eliminate the absolute Raman intensity effects. A common way of normalization is to divide the spectrum by the most intense peak, which is the band at 1000 cm^{-1}. However, this band is significantly affected or destroyed by fungi growth. Its intensity changes drastically from one sample to another. By visually inspecting the spectra we concluded that peaks corresponding to C-H stretching vibrations in the region of 2850-3030 cm^{-1} are not quite sensitive and stayed more stable against the effects of fungi growth. For this reason, we chose C-H stretching peak at 2940 cm^{-1} as an internal standard and normalized each spectrum to its intensity. Figure 11 shows the spectra in which background is removed and normalized. Each spectrum is offset on the ordinate for a better clarity.

It is evident from their Raman spectra in Figure 11 that control and *P. chrysogenum* inoculated samples both yielded very similar spectral signatures. On the other hand, *P.glabrum, P.echinilatum, P. brevicompactum* and *P. commune* inoculated samples produced major differences from control sample,

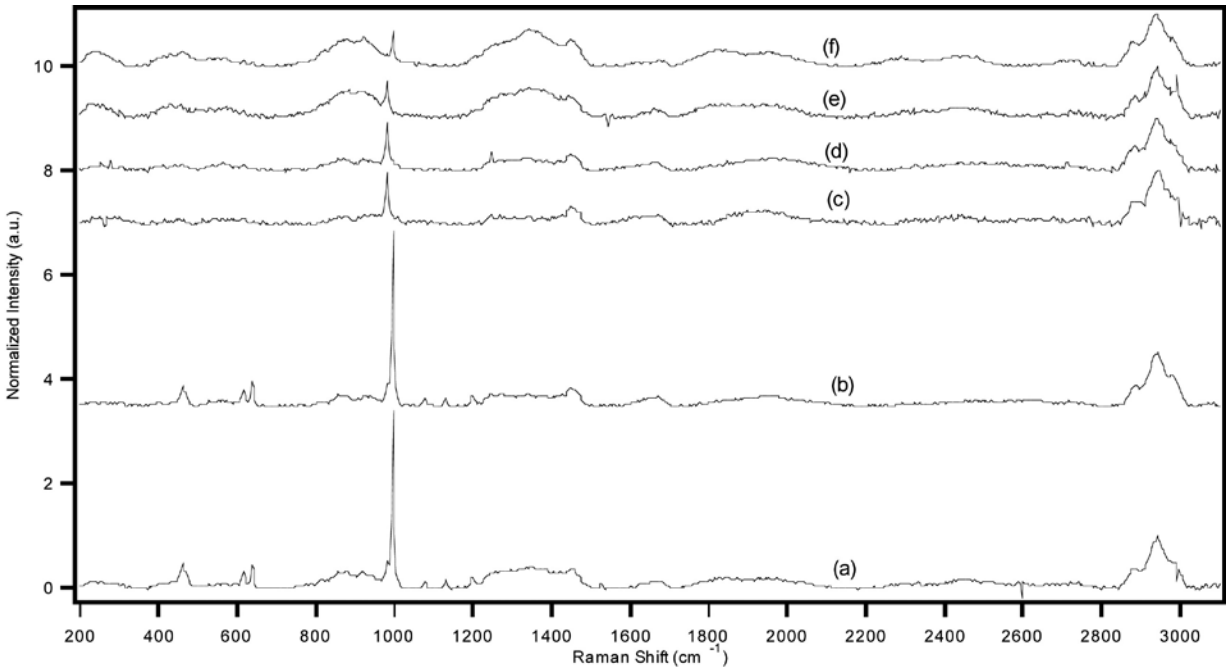

Figure 11. Fluorescence removed and normalized Raman spectra. Each spectrum is offset on the ordinate.
(a) Control; (b) *P. chrysogenum*; (c) *P. glabrum*; (d) *P. echinilatum*; (e) *P. brevicompactum*; (f) *P. commune*

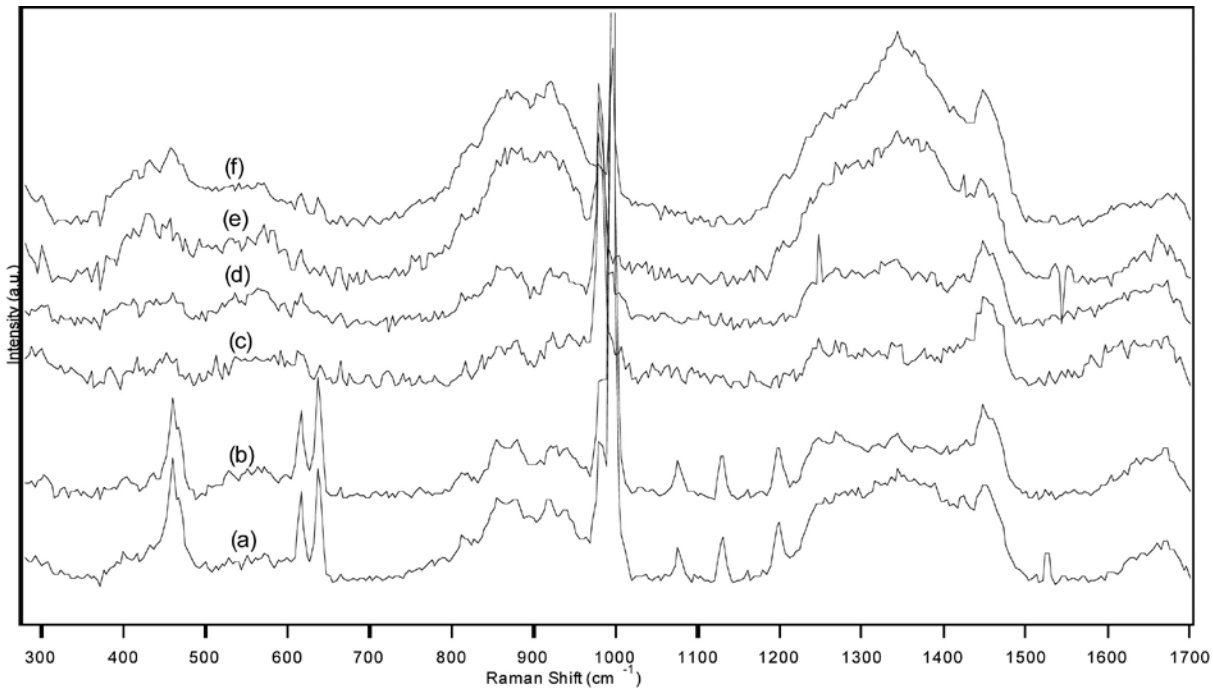

Figure 12. (a) Control; (b) *P. chrysogenum*; (c) *P. glabrum*; (d) *P. echinilatum*; (e) *P. brevicompactum*; (f) *P. commune*

showing that these *Penicillium* species caused structural changes on the leather. Figure 12 is constructed to be able to better analyze the spectral differences among the samples. Only the region between 350 and 1600 cm^{-1} is shown in Figure 12 (A) and the peaks at 1000 cm^{-1} are truncated on y axis for better inspection of lower intensity bands. Careful inspection of the control and *P. chrysogenum* spectra in Figure 12 (A) shows slight differences. However, these changes caused by *P. chrysogenum* are very subtle changes from control sample when the changes caused by *P. glabum, P. echinilatum, P. brevicompactum* and *P. commune* are considered. Both control and *P. chrysogenum* spectra show the same intensity and shape for all major peaks, specifically the band at 1000 cm^{-1} and singlet and doublet at 460 cm^{-1} and 624 cm^{-1} respectively. However, the spectra of *P. commune, P. echinilatum, P.brevicompactum* and *P. glabrum* show major differences in the same regions. Figure 12 (B) shows only the peaks in 1000 cm^{-1} region. As it is seen in Figure 12 (B), the peak at 1000 cm^{-1} did not change in *P. chrysogenum* spectrum compared to control sample. On the other hand, the intensity of this same peak in *P. commune* spectrum decreased significantly to 19% of that of control sample, and it virtually disappeared in *P. echinilatum, P. brevicompactum* and *P. glabrum* spectra. Also, the singlet at 460 cm^{-1} and doublet at 624 cm^{-1} region disappeared in *P. glabrum, P. commune, P. echinilatum,* and *P. brevicompactum* spectra but conserved in *P. chrysogenum* spectrum (Figure 12).

CONCLUSION

We demonstrated the potential of Raman spectroscopy to investigate the spectral changes on wet blue sheep leather caused by various fungi from the genus *Penicillium*. Our Raman results indicated that of all the fungi species we studied, *P. chrysogenum* least affected the structure of the wet blue sheep leather. To the best of our knowledge, this is the first study in this regard. This study will be an important starting point, especially for biocidal agent manufacturing companies, because we believe that the microorganisms that damage the leather structure would be the most undesired ones, which effective measures should be taken against. Although broad spectrum effect of a fungicide is given attention during the manufacturing process, fighting with a microorganism can be unnecessary unless it damages the leather. The use of Raman Spectroscopy to identify this damage may be one of the methods to be implemented. A fungus or its enzymes which does not cause structural changes on the leather but at the same time has an antagonistic effects against other fungi that would damage the leather, can be used as an agent for biological fight against the harmful fungi. We believe that this study will also be a forerunner for those who are willing to pursue research on this matter.

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