Fungal growth on wetblue:
Methods to Measure Impact on Leather Quality

By

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Abstract:
Wild and known fungal species were selected to inoculate a standardized wetblue leather and various methods were then employed to quantify the concomitant damage associated with mold growth. Fungi were isolated from commercially tanned samples of contaminated wetblue and pure strains were sourced from ATCC cultures. Test cuttings of wetblue leather were inoculated with the selected cultures and incubated along with matched control cuttings for a period of 90 days. Unknown wild strains were later identified using DNA sequencing techniques. Microscopic observations of all treated versus control samples did not show any difference in grain structure or skin morphology, indicating little or no breakdown of intact and tanned collagen structures. Various staining and analytical assay techniques indicated significant reduction in fatty material content in the treated samples compared with the untreated controls.

Key Words: Fungal identification, Gene sequencing, API®ZYM, Wetblue, Staining, Leather

1. INTRODUCTION

It is universal practice to add a fungicide during any steps in the leathermaking process where leathers (wetblue, wet white, vegetable tanned, or crust) will be held or stored in a wet or moist condition for a period of time. The fungicide is necessary to protect the leather against attack by fungi as the leather contains adequate nutrition and industry environmental conditions are typically favorable for fungal growth. Airborne spores are everywhere, and fungal growth can occur within days unless wet leathers are adequately protected. Tanners are well aware that such growth is damaging to the leather surface and can result in significant direct and indirect costs such as staining, non-uniformity in further processing, grain damage, changes in physical parameters, organizational costs related to re-work, and customer complaints. Tanners are less aware of the potential costs linked to worker health. High levels of fungal spores can elicit short term allergic response. Some of the common strains of fungi identified in the leather industry are also known to produce mycotoxins under certain conditions which can have more severe and longer term consequences [1].

In reviewing the literature regarding fungi and the damage it does on leather, it is apparent that much of the systematic work was done many years ago, mainly in the USA, and these studies focused on finished leathers [2 – 6]. This work is helpful as it clearly linked the growth of mould to loss of lipids and deterioration of the physical strength properties. The body of work was well summarized in 1965 by T.C. Cordon [7]. More recent work has focused on identification of different species growing on various leathers [1, 8-10] and the benefits of various fungicides and their ability to control mould species. While these recent articles reference some of the damage done by molds, there is no systematic attempt to document the extent of damage done by a particular species and linking that to specific changes in components of the leather substrate.

It is also relevant to note that much of the prior work done in identifying fungal species growing on leather did not have the benefit of the modern gene sequencing techniques which are known to be accurate, specific, and much faster [11].

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This work focuses on the development of methods that would allow a systematic study of some of the types of fungal species found growing on commercial wetblue and relating that to the specific damage done by these microorganisms on components of the leather substrate.

2. EXPERIMENTAL

2.1 Leather sample preparation, inoculation, and fungal growth

The raw material used was frozen baby calf to ensure minimum damage was present on the grain surface. The skins were processed using standard unhairing and chrome tanning techniques. The skins were cut into sample squares as shown in the Diagram 1. A total of 24 sets of coupons were prepared. Each coupon was then marked with a unique series of holes and cuts for identification and alignment. It was then cut into four equivalent pieces to provide two Test and two Control pieces each of dimensions 5cm x 5cm. This yielded 96 squares of leather for evaluation.

Diagram 1: Coupon Preparation with Control and Test Pieces marked as shown.

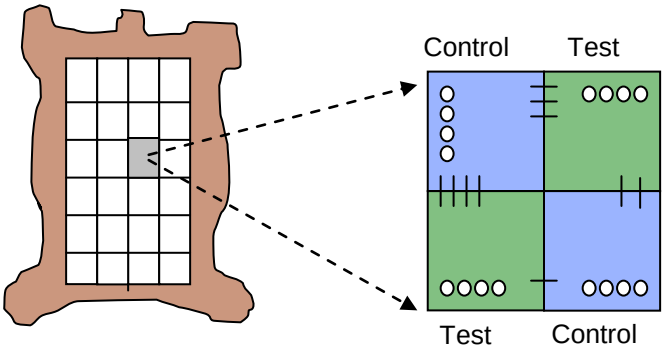

Fig. 1: Coupon after incubation, showing the two Test areas (BTL-5) with fungal growth and two control areas with no growth (BCL-5).

Both test and control pieces were washed separately with 200% water at 30ºC for 45 minutes. To each of the control drums were added 0.2% fungicide[1]. A folded Whatman #1 filter paper was placed at the bottom of each of 96 Petri Dishes and 2.5 ml of sterile distilled water added to help retain moisture during the incubation period. The leather pieces were placed on the filter paper and test pieces (cut 1/4 & 2/3) were inoculated in duplicate with designated fungal spores and covered. The corresponding control pieces (cut 1/2 & 3/4) were covered without inoculation. The dishes and leather pieces were incubated at room temperature for 90 days (See Fig. 1). Periodic addition of sterile water to the filter paper was made to ensure that sufficient moisture was present to facilitate growth. No nutrients were added.

Table 1: Coupon test pieces inoculated with wild and known strains (*ATCC).

BTL 1	*Arthrobotrys amerospora**	MTL 1	*Aspergillus sp.*
BTL 2	*Arthrobotrys tortor**	MTL 2	*Wild Penicillium sp.* – Australia
BTL 3	*Aspergillus versicolor**	MTL 3	Wild Species - US Trop chamb.
BTL 4	Wild species – China	MTL 4	Wild Species - US Trop chamb.
BTL 5	Wild species – Europe	MTL 5	*Aspergillus niger**
BTL 6	Wild species – Europe	MTL 6	*Aspergillus terreus**
BTR 1	*Arthrobotrys tortor**	MTR 1	*Trichoderma viride*
BTR 2	*Penicillium sp.*	MTR 2	Wild species – Europe
BTR 3	*Aspergillus versicolor**	MTR 3	Wild species – Europe
BTR 4	Wild species – USA	MTR 4	*Trichoderma viride*
BTR 5	Wild species – China	MTR 5	*Paecilomyces nivea*
BTR 6	*Arthrobotrys amerospora**	MTR 6	*Penicillium* or *Paecilomyces*

2.2 Physical Observations

Optical microscopy evaluations of the grain and stained cross-sections were done using various standard laboratory visual and stereoscopic light microscopes. Electron microscopy evaluation and pictures (SEM) were done with Jeol JSM-6480LV, at 15 to 20 kV, low vacuum. A flat pH probe was used to determine if there were measurable differences in surface pH.

2.3 ***Staining techniques***

Following the incubation period, the surface of each wetblue piece, along with corresponding control, was briefly rinsed with water to remove adhering surface mould. The grain surface was inspected and cross-section specimens of the matched and aligned Test and Control wet blue pieces were prepared using a radial microtome and cryostat method. The pieces were then stained using the following techniques:

2.3.1. Sudan IV solution for natural fats, oils and lipid materials was prepared according to Tancous [12], using Sudan IV Sigma-Aldrich Cat 94261, St. Louis, MO, 63103, USA.

2.3.2. Sudan Black solution for natural fats, oils and lipid materials was prepared as for Sudan IV, using Sigma-Aldrich Sudan Black Cat 199664, St. Louis, MO, 63103, USA.

2.3.3. Gentian Violet stain for soluble protein materials was prepared according to Tancous [12], using Accustain Crystal Violet solution Sigma-Aldrich Cat HT901, St. Louis, MO, 63103, USA.

2.3.4. Elastin stain was made using Sigma Accustain Elastin Stain kit procedure HT25, Sigma-Aldrich Cat 94261, St. Louis, MO, 63103, USA.

2.4 Fungal Identification using DNA sequencing technique

Some of the test leather pieces showing fungal growth were selected and fungal samples removed and grown on potato dextrose agar supplemented with chloramphenicol. Isolated pure cultures were used for fungal identification using the MicroSeq D2 LSU ribosomal DNA Fungal Kit (Applied Biosystems, Foster City, CA, USA). In cases where there was no match with the Applied Biosystems' microbial identification database, a BLAST search of the GenBank was conducted.

2.5 Enzyme profile of fungi

Samples of three known fungi showing good growth on three different leather samples were carefully removed from the surface using a scalpel and then conditioned in 5ml of a lightly buffered solution at pH 7.0. An aliquot was then transferred to each of 20 slots of the API®ZYM kit, Bio Mérieux SA, Lyon, 69280, France. The test strip was left overnight at room temperature. In the morning, reagents were added to react with converted substrate and the strips then exposed to sunlight for 10 minutes to develop the color. Specific enzyme identification was made by comparison to the control as described by the manufacturer.

2.6 Fat analysis

Sixteen wetblue paired samples were extracted in dichloromethane following standard procedure (IUC-4).

3. RESULTS & DISCUSSION

3.1. Wetblue sample preparation and fungal growth

The use of young unblemished calfskin and the sample preparation techniques allowed a direct side-by-side comparison of identically treated wetblue leathers, thereby ensuring that any physical or chemical differences seen are directly attributable to the growth of mould and not to other factors. However, it is suggested for future testing that the skins should be fully fleshed to

improve uniformity. The inoculation and growth of fungi was observed on a bi-weekly basis and although some drying out of the samples occurred, growth on all the inoculated pieces was significant. There was no growth on any of the control pieces treated with fungicide.

3.2. Physical Observations

Visual inspection showed the mold growing mainly on the grain surface. There was minimal growth over the cut sides of some pieces and occasional growth on the flesh side in contact with the filter paper or base of the Petri dish. In all cases, the growth of the hyphae was only observed on leather surfaces, with no penetration into the interior of the skin cross-section indicating that growth of mould in wet leathers is a surface phenomenon only.

Initial observations of the integrity of the grain surface of the samples were made using an optical microscope and a stereoscope. No difference in grain structure or skin morphology was apparent on any of the test versus control samples when adjacent pieces were carefully aligned to compare surface or cross-section. This indicates little or no breakdown of intact and tanned collagen structures.

The samples were also evaluated using SEM under magnifications ranging from 40x to 600x. Careful observation revealed no observable structural or morphological differences between the test versus control (see Fig. 2).

The surface color of the test leathers was a darker shade than the control. There were no statistical differences in pH between the test versus control wetblue that could be measured using a surface pH probe (no data shown).

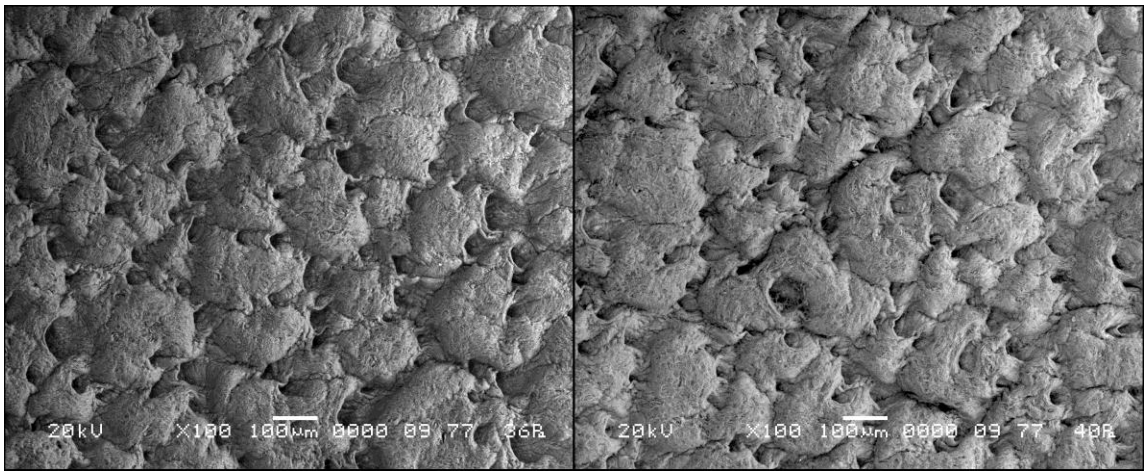

Fig.2. Sample BTL-5 observed at 100x. The left side is test and right side control

3.3. Staining on the wetblue

When stained with Sudan IV and Sudan Black, the fat deposits were clearly seen and the results observed were consistent for all samples. The control (no mold) stained more intensely than test (molded) wetblue, indicating significant removal of natural fats, oils and lipids in the test pieces. It was further observed that although the fungal growth was almost exclusively superficial and on the grain surface, the reduced fat levels extended through the grain and to the flesh side (Fig. 3 – 5). The staining results using Gentian Violet and elastin indicated some removal of soluble protein and elastin in some samples, but the results were not consistent in all test pieces.

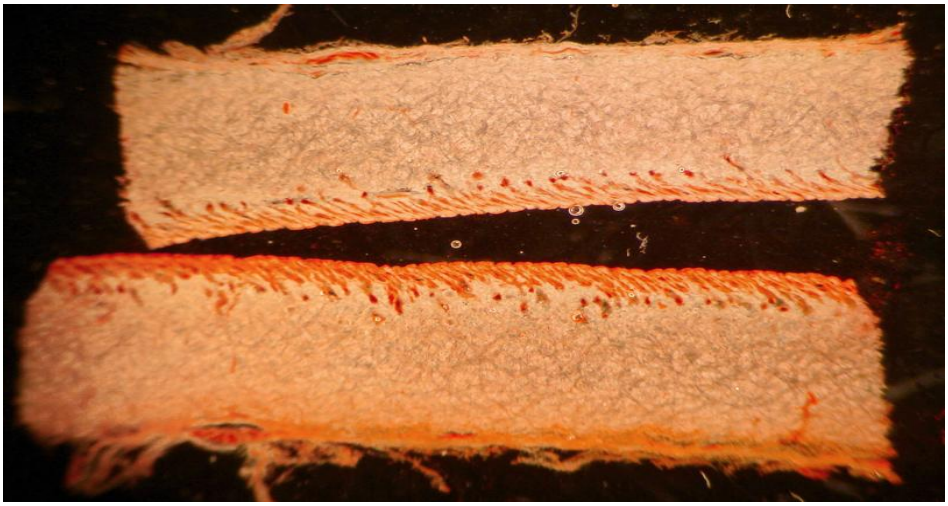

Fig.3. Sample BTR1 test on top and BCR-1 control on bottom showing full cross-section stained with Sudan IV to reveal fats. More intensely colored control indicates a higher residual fat content.

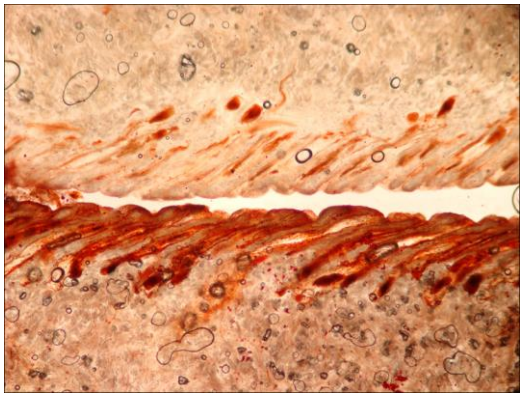

Fig.4. BTR-1/BCR-1 with Sudan IV at higher resolution showing depletion of fat on the upper test grain surface.

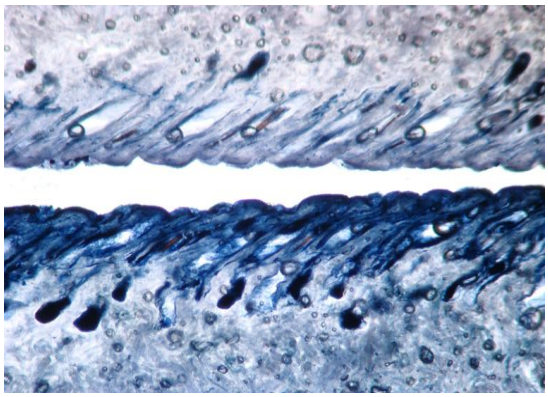

Fig.5. BTR1/BCR1 with Sudan Black confirming depletion of fat on upper test grain surface.

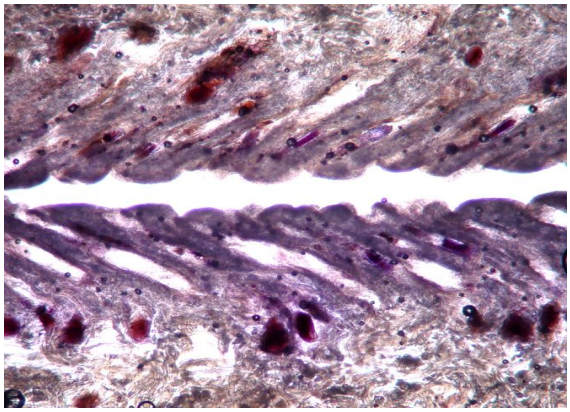

Fig.6. BTL-1/BCL-1 with Gentian violet. This general protein stain consistently showed reduced soluble protein in the upper test with more intensely stained lower control.

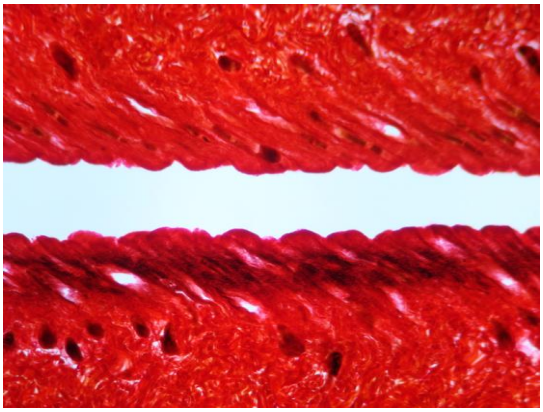

Fig. 7. BTL-1/BCL-1 with stain for elastin. This specific cross-section shows the grain more darkly stained in the lower control and elastin depletion in the upper test.

3.4. Fungal Identification using DNA Identification Technique

3.4.1. Sample MTL4 - one fungus was isolated and identified as *Trichoderma harzianum.*

3.4.2. Sample BTL6/BTL5 - two fungi were isolated and identified as *Trichoderma atroviride* and *Penicillium sp*. The closest species match for the later isolate was *Penicillium rubrum* (92.49%).

3.4.3. Sample BTL4 - one fungus was isolated and identified as *Penicillium sp.* The closest species match for the later isolate was *Penicillium solitum* (99.10%).

This technique allows rapid and accurate identification of fungal species, provided the nucleotide sequence exists in the gene database. For the two *Penicillium* species found growing on wetblue leather, there was not a good species match in the databases.

3.5. Enzyme profile of selected known fungi

Fig 8. API®ZYM racks with enzyme assays. Top to bottom - A*spergillus niger, Aspergillus terreus* and *Paecylomyces nivea*

Table 1: Enzyme assay results (+ positive result; blank = negative result)

No.	**Enzyme in Well**	***Aspergillus niger***	***Aspergillus terreus***	***Paecylomyces nivea***
1	Control			
2	Alkaline phosphatase			
3	Esterase (C4)	+	+	+
4	Esterase lipase (C8)	+	+	+
5	Lipase (C14)			
6	Leucine arylamidase	+	+	+
7	Valine arylamidase		+	
8	Cystine arylamidase			
9	Trypsin			
10	α-chymotrypsin			
11	Acid phosphatase	+	+	+
12	Naphtol-AS-Bl-phosphohydrolase	+	+	+
13	α-galactosidase		+	
14	β-galactosidase			
15	β-glucoronidase			
16	α-glucosidase			+
17	β-glucosidase	+	+	+
18	N-acetyl-β-glucosaminidase	+	+	+
19	α-mannosidase			
20	α-fucosidase			

Between 7 and 9 enzyme types were positively identified using the API®ZYM technique on scrapings of molds removed from the test leathers. The enzymes were predominantly lipases and glycosidases as seen in the assay results (Fig. 8 & Table 1). Further work should be undertaken to identify additional proteolytic enzyme activity.

3.6. Fat analysis

The observations on staining with Sudan IV and Sudan Black gave a good indication that molded test wetblue samples had significantly reduced fat content compared to the control wetblue. This observation was also manifested during sample preparation for fat analysis where air dried test wetblue showed grain crack (Fig.9) while the control did not. Results of the fat extractions of 8 matched samples of test and control are shown in the Figure 10. On average the test had less than 50% of the total fat present compared to adjacent control pieces.

Fig 9: Air dried wetblue showing grain rupture on the test (molded leather) and intact control

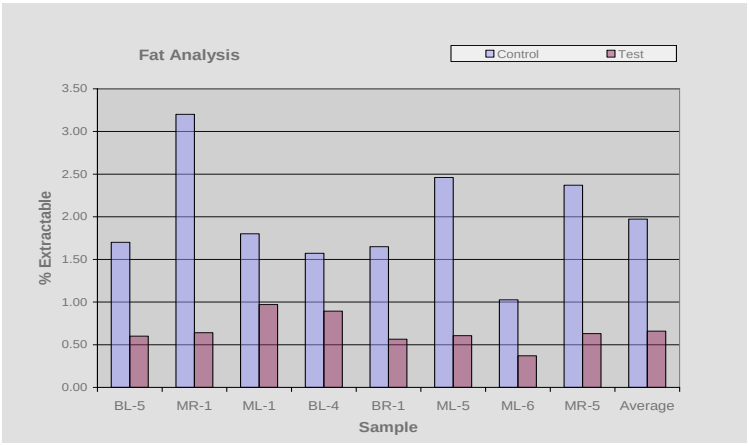

Fig. 10: Fat analysis of matched control versus test wetblue samples

4. CONCLUSIONS

The major difference between the test and control pieces was the large reduction of fatty materials as seen in staining techniques and fat analysis. Oil, fat and lipid materials appear to be the major components degraded by fungi growing on wetblue leathers. What was surprising was the extent of removal of fats through the cross-section. This would suggest significant penetration of lipolytic enzymes through the leather. The significantly reduced fat content also explains the resulting loss in physical strength properties documented in leathers that have sustained severe mold damage. It is anticipated, based on industry experience that this removal of fat could also result in physical and aesthetic changes that are irreversible.

For growth, fungi require additional nutrients, including nitrogen. It is suggested that these may come from inter-fibrillar non-structural proteins such as residual glycosaminoglycans or the serum proteins not removed during processing. As some nutrients are only required in very small amounts, they could also come from residual process contaminants. There was no evidence of any destruction of intact tanned structural proteins.

This study provides a good basis for further systematic study of the potential damage done by defined species of fungus on wetblue leather and leather components. The loss of fatty material

is possibly the major contributor to the damage done by fungi growing on wetblue. The extent of fatty material loss will impact aesthetic and physical properties of crust or finished leather. Further work is needed to confirm the extent of any damage to tanned structural fibers, such as collagen or elastin, and other leather components.

5. REFERENCES

[1] M. Birbir; O. Ozyaral; C. Johansson; A. Ilgaz. JALCA, 1994, 89: 14-19.
[2] J.R. Kanagy; A.M. Charles; E. Abrams; R.F. Tener. JALCA, 1946, 46: 198-213.
[3] J.R. Kanagy; R.E. Seebold; A.M. Charles; J.M.Cassel. JALCA, 1949, 44: 270-282.
[4] E.S. Barghoorn. JALCA, 1950, 45: 688-700.
[5] H.R. Wilson; H.B. Merrill. JALCA, 1954, 49: 404-407.
[6] A.J. Musgrave, R.G. Mitton. JSLTC, 1950, 34: 324
[7] T.C. Cordon: O'Flaherty, Roddy & Lollar. Chemistry & Technology of Leather, 1965, (4) 65: 333-368
[8] O.P. Sharma; K.D. Sharma. Def. Sci. J., 1977, 27: 81-82.
[9] O.P. Sharma; K.D. Sharma. Def. Sci. J., 1979, 29: 77-78.
[10] A. Orlita. Int. Biodeterioration & Biodegradation, 2004, 157-163.
[11] D. Oppong; S.D. Bryant; R. Rangarajan; S. Steele. JALCA, 2006, 101: 140-145.
[12] J.J.Tancous, Skin, Hides and Leather Defects, Lee Corporation, Cincinnati, OH, 1986, p. 329

[1] Product Reference: Busan 30L from Buckman Laboratories and prepared as a 1:20 emulsion.