BIOTECHNOLOGY FOR ENVIRONMENT-FRIENDLY LEATHER PRODUCTION

J. Y. Liu[1,a] and G. Holmes[1]

1 New Zealand Leather and Shoe Research Association

Fitzherbert Science Centre Dairy Farm Road, Palmerston North 4414, New Zealand

a) Corresponding author: john.liu@lasra.co.nz

Abstract. LASRA research is guiding the application of biotechnology to help the New Zealand leather industry develop environmentally sustainable leather processes. Using 16S rRNA gene sequencing, we have isolated and identified a number of indigenous bacteria from the leather industry environment which are being adopted to develop benign leather processing technologies. We isolated and identified several *Bacillus* strains from a biofilter used in a leather manufacturing plant which exhibited sulphide oxidation activity, which are being applied in bioremediation of volatile organosulphur compounds emitted by leather products. We also discovered a strain of *Stenotrophomonas* spp. with significant and beneficial proteolytic activity in a tannery sludge. The identified strain not only displays collagenase activity but also the ability to reduce hexavalent chromium to trivalent chromium, making it an ideal candidate for biodegradation of tanned waste. Recently we revisited the natural autolytic processes of degradation of untreated pelts to guide a natural depilation method without any need for additional chemical treatment. In controlled experiments the wool could be removed completely from follicle after 2 days, without obvious damage and leathers could be processed with mechanical properties comparable to conventionally processed counterparts. The alkaline protease activity of the isolated bacteria is responsible for the observed natural unhairing.

1 Introduction

Leather production not only serves social needs by utilising a meat by-product such as hide and skin, but also contributes significantly to the global economy through trade and employment. But the social image of the leather industry has become negative with increasing awareness of environmental protection and the demand among consumers for products made in a sustainable way with less environmental impact. In line with New Zealand's global reputation of being an eco-friendly country, the New Zealand leather industry has committed to improving its environmental performance and image by adopting sustainable practice throughout the production process and providing high quality leather products with reduced environmental impact during their life cycle (Collins, Roper & Lawrence, 2010; Zhang, et al., 2018). Chemicals extensively used during leather production have also been regarded as a source of unpleasant odour sometimes emitted from leather products, especially in cabin upholstery (Wang, Ma, Chen, Sun & Fan, 2018). In certain regional markets, such as Asia, consumer concerns about the indoor or in-cabin air quality relate odour emission with health threats, which impacts their purchase decisions on leather products (Pan, Walsh, Dearth & Zhang, 2017). Besides consumers' preference, the odour has been proved to be caused mainly by volatile organic compounds (VOCs) which are indeed harmful at high concentrations (Dai, et al., 2017). Concerns for indoor air quality and consumers' health have driven the implementation of legal regulations applicable to the emission limits of VOCs from products, including leathers, which are becoming increasingly strict (Xu, Chen & Xiong, 2018). Therefore, the development of leathers with low VOC emissions will not only ensure improved market acceptance, but also contribute to a healthier indoor environment.

Biotechnology such as enzymatic processing has been considered a realistic substitute for current leather production practice (Thanikaivelan, Rao, Nair & Ramasami, 2004). Proteolytic enzymes including alkaline protease and keratinase have been investigated for their potential to replace the large quantities of chemicals used in the unhairing step, which is the most polluting operation (Rao,

et al., 2017; Giongo, Lucas, Casarin, Heeb & Brandelli, 2007). Since the raw materials used and waste generated during leather production are generally proteinous, the leather industry environment could serve as an ideal resource for the discovery of microbes producing useful proteolytic enzymes. Guiding the application of sustainable biotechnologies to assist the transition of the New Zealand leather industry (Naffa, Edwards & Norris, 2019; Naffa, et al., 2019), LASRA has developed the capability to identify potentially valuable microorganisms from the local industrial environment including storage, sludge, compost, etc. The identification of bacterial species is carried out routinely at LASRA by 16S rRNA gene sequencing, which has been widely applied to study bacterial phylogeny and taxonomy, as this conservative gene is believed to be the most common housekeeping genetic marker in almost all bacteria (Langille, et al., 2013). LASRA is also establishing platforms to characterise and produce useful microbial enzymes, which could be applied in alternative leather processes. While cleaner enzymatic processes are still being developed, issues related to VOCs emitted from leather products can be addressed by deploying VOC consuming bacteria during conventional leather production. As the oldest biological method for removal of undesirable gaseous compounds from air, biofiltration uses microorganisms as the engine of the biotreatment process (Delhoménie & Heitz, 2005). Because biofilters operate as fixed-bed bioreactors with immobilised active microorganisms, it is reasonable to hypothesize that VOC metabolising-bacteria can be isolated from biofilters dealing constantly with such pollutants, such as those used by tanneries.

In the present study, bacteria with the potential to benefit sustainable leather production were isolated from the New Zealand leather industry environment. Isolated strains were identified using 16S rRNA gene sequencing. A preliminary investigation of the impact of bacterial activity on leather performance was also carried out. Detailed characterisation of the properties of the identified bacterial strains, such as VOC metabolisation, hexavalent chromium reduction, enzymatic activity and substrate specificity, etc. will be reported elsewhere separately.

2 Materials and Methods

2.1 Bacteria isolation

Bacteria with odour mitigation potential were isolated from the soil bed of a biofilter used by a New Zealand tannery. A sample of the soil was mixed with phosphate-buffered saline (PBS) solution to prepare a 10% (w/v) suspension. Serial dilutions of the soil suspension were plated onto LB agar plates, which were then incubated at 37 °C. Colonies with visually distinguishable morphology were selected for further studies.

Bacteria with proteolytic potential were isolated from sludge and sheepskins. Sludge samples collected from LASRA's tannery waste treatment plant were suspended in PBS to prepare 10% (w/v) suspensions. Sheepskins were freshly provided by a local slaughterhouse and cut into halves along the backbone. The right halves were depilated conventionally as a control, and the left halves were kept at ambient temperature until the wool could be manually removed. A piece of skin sample was then taken from each left half and a bacterial suspension was prepared by placing each skin sample in a 50 mL centrifuge tube containing 20 mL PBS solution shaken at room temperature for 4 hours at 200 rpm. Serial dilutions of the bacterial suspensions were plated onto LB agar plates with 2.5% (w/v) skimmed milk at 37 °C. The proteolytic activity was detected by the formation of translucent halos around the individual colonies, which was a result of the hydrolysis of casein in the milk.

2.2 Bacterial identification

Identification of the isolated bacterial strains was carried out by 16S rRNA gene sequencing. The candidate colonies were picked up and cultured in 5 mL LB broth medium for 24 h at 37 °C. Genome

DNA from each culture was extracted and 16S rRNA genes were amplified by PCR using primer pair 27F and 1427R. DNA electrophoresis with 2% agarose gel was employed to verify the successful amplification of the 16S rRNA genes from all the candidate colonies. The PCR products were purified before being submitted to Massey Genome Service for sequencing. The sequencing results were analysed using the Targeted Loci Nucleotide BLAST and the phylogenetic trees were constructed using the Neighbour-Joining method with p-distance.

2.3 Physical Properties of Crust Leathers

Sheepskins were processed into crust leathers following LASRA's standard protocol. Physical properties examined included tear strength, tensile strength and percentage elongation at break, and grain crack resistance. Comparison was made using 4 samples from each half of skin and each group consisted of 3 skins.

3 Results and Discussions

3.1 Bacteria with odour mitigating potential

The biofilter facility used by a New Zealand leather manufacturer was found to be supportive of the growth of plants within it. The air emitted from the production hall after filtration though the soil bed did not provoke any noticeable perception of unpleasant odour. From the soil sample collected in the biofilter 4 *Bacillus* species were identified as potential candidates responsible for the removal of odorous compounds during filtration of the gaseous waste emitted by the leather manufacturer.

These bacteria have been reported to promote plant growth mainly through nitrogen fixation (Yousuf et al., 2017), which was consistent with the overall observation of the biofilter facility where healthy plants were thriving across the entire soil bed of the biofilter. These species have previously been characterised as being interactive with sulfur and ash from coal (Abdel-Khalek & El-Midany, 2013), indicating their potential application in the bioremediation of leather odour by metabolising the sulfur-containing volatile compounds emitted from leather products. In other research at LASRA, the volatile compounds emitted by New Zealand leather products have been profiled to identify the odorous molecules, serving as targets to be mitigated by bioremediation (data not shown). The metabolism of the identified compounds by the *Bacillus* species isolated in this study has been examined to reveal the responsible bioremediation mechanisms and the results of this will be reported separately soon. Comparison between the volatile profile of leather products before and after bacterial treatment will demonstrate the efficacy of the proposed strategy for leather odour mitigation. Additionally, the identified species strongly inhibit the growth of pathogenic fungi across a wide range of host plants (Liu, Wei, Zhu, Du & Feng, 2008). Therefore, in addition to reducing unpleasant leather odours, the identified stains might be applied to develop novel biocontrol methods, to prevent damage to leather caused by fungi.

3.2 Proteolytic Bacteria in Tannery Sludge

Consistent with LASRA's previous finding on the proteolytic potential of tannery sludge, the present study isolated and identified the responsible bacterial strain from the sludge treatment. From 16S rRNA gene sequencing and phylogenetic analysis, the dominant strain in the tannery sludge exhibited 99% sequence identity with a *Stenotrophomonas* strain.

Members of the genus *Stenotrophomonas* have been reported to have keratinase activity (Fang, Zhang, Liu, Du & Chen, 2015) and also to reduce hexavalent chromium to benign trivalent chromium (Raman, Asokan, Sundari & Ramasamy, 2018). Our preliminary experiments have revealed that the

identified strain exhibits collagenase activity as well as tolerance to hexavalent chromium (data not shown). These results suggest that this strain might usefully contribute to the biodegradation of tanned leather waste. Currently we are optimising the enzyme production conditions and the bioremediation of hexavalent chromium. The activity of enzymes produced by the identified strain, including collagenase, keratinase, lipase, neutral protease, and alkaline protease are currently being characterised. The application in beamhouse operations of proteases produced by the identified strain is also being investigated with promising progress and the results will be reported soon.

3.3 Bacteria Responsible for Natural Wool Loosening

The natural wool loosening induced by microbial protease has attracted interest for a long time (Green, 1955). In the present study, it was found that after storage for 48 hours, the wool throughout the skin could be removed by pulling effortlessly, leaving empty follicles and a slightly damaged grain. Proteolytic bacteria on the skins which might be responsible for the observed natural wool loosening were identified as *Aeromonas* spp, *Proteus* spp, and *Wohlfahrtiimonas* spp.

Aeromonas spp has long been known to produce extracellular proteolytic enzymes (O'Reilly & Day, 1983). *Proteus* species secret protease as one of the virulence factors associated with the infection process and disease (Yu et al., 2017). Among strains of *Proteus* spp., one identified in this study has previously been found to be present as one of the bacterial strains causing wool loosening (Maxwell & Lennox, 1944). *Wohlfahrtiimonas* spp has been demonstrated to be associated with myiasis, infection with the larvae of parasitic flies (Campisi, Mahobia, & Clayton, 2015). The strong chitinase activity of *Wohlfahrtiimonas* spp may play a role in the metamorphosis of the fly (Schröttner et al., 2017). *Lucilia sericata* larvae linked to the infection of *Wohlfahrtiimonas* spp are used as an alternative treatment for recalcitrant and chronic wounds, which could be attributed to the various peptidases within the excretions secretions (Franta et al., 2016). While the effect of chitinases on the depilation of sheepskin is still inconclusive, the proteolytic enzymes from maggots might contribute to the natural unhairing process.

In a subsequent study, the protease activity and the substrate specificity of the enzymes secreted by the identified strains has been determined and will be published soon. The potential enzyme candidate can be produced by fermentation and applied, experimentally, replace lime and sodium sulphide in the depilation process. The methods developed using sheepskin as a model can also be adopted on other materials such as cow hides. The noteworthy bacteria-associated maggots' activities will be investigated to enhance biodegradation of leather waste.

3.4 Physical properties

The crust leathers obtained from conventional chemical depilation and natural wool loosening were examined for their physical properties. As shown in Table 1, leathers produced from natural processing presented comparable strength properties to their chemically processed counterparts, such as tear strength, tensile strength, elongation. It is noteworthy that the grain crack resistance of the leathers processed with natural wool loosening is significantly high than that of the leathers processed traditionally. The improvement in the physical characteristics of leather processed from skins with natural wool loosening might be attributed to improved uptake of tanning chemicals which has been observed in enzymatically processed leather (Ranjithkumar, Durga, Ramesh, Rose, & Muralidharan,2017).

Table 1. Physical properties crust leathers.

Group	Tear strength (N/mm)		Tensile strength (N/mm^2)		% Elongation at break		Grain load (N)	Grain extension (mm)
	Parallel	Perpendicular	Parallel	Perpendicular	Parallel	Perpendicular		
Chemical	31.0 ± 3.8	24.6 ± 8.6	11.7 ± 0.77	5.9 ± 0.7	50.3 ± 17.8	175.3 ± 11.5	6.1 ± 3.2	6.0 ± 0.4
Natural	27.0 ± 6.4	17.5 ± 6.2	14.2 ± 3.27	5.0 ± 2.3	47.1 ± 8.2	140.1 ± 67.9	39.8 ± 5.4*	11.1 ± 0.9*

* indicates statistically significant differences ($p < 0.05$).

4 Conclusions

Our research is aimed at enabling the New Zealand leather industry to produce high quality leather products with a much-reduced environmental footprint. The application of biotechnology helps the leather industry adopt a more sustainable practice. Our results demonstrate that the VOC profile of leather products can be improved by treatment with bacteria isolated from a biofilter. The bacteria isolated from sludge treatment presented protease activity, which is being investigated for biodegradation of leather waste as well as enzymatic depilation of hide and skin. Extracellular protease producing bacteria were isolated from locally-sourced sheepskins. The crust leathers processed from skins influenced by those bacteria presented comparable or even improved physical properties, compared with their conventionally-processed counterparts.

References

1. *Abdel-Khalek, M. A., & El-Midany, A. A. (2013). Application of Bacillus subtilis for reducing ash and sulfur in coal. Environmental earth sciences, 70(2), 753-760.*
2. *Campisi, L., Mahobia, N., & Clayton, J. J. (2015). Wohlfahrtiimonas chitiniclastica bacteremia associated with myiasis, United Kingdom. Emerging Infectious Diseases, 21(6), 1068.*
3. *Collins, E., Roper, J., & Lawrence, S. (2010). Sustainability practices: trends in New Zealand businesses. Business Strategy and the Environment, 19(8), 479-494.*
4. *Dai, H., Jing, S., Wang, H., Ma, Y., Li, L., Song, W., & Kan, H. (2017). VOC characteristics and inhalation health risks in newly renovated residences in Shanghai, China. Science of The Total Environment, 577, 73-83.*
5. *Delhoménie, M. C., & Heitz, M. (2005). Biofiltration of air: a review. Critical reviews in biotechnology, 25(1-2), 53-72.*
6. *Fang, Z., Zhang, J., Liu, B., Du, G., & Chen, J. (2015). Insight into the substrate specificity of keratinase KerSMD from Stenotrophomonas maltophilia by site-directed mutagenesis studies in the S1 pocket. RSC Advances, 5(91), 74953-74960.*
7. *Franta, Z., Vogel, H., Lehmann, R., Rupp, O., Goesmann, A., & Vilcinskas, A. (2016). Next generation sequencing identifies five major classes of potentially therapeutic enzymes secreted by Lucilia sericata medical maggots. BioMed research international, 2016.*
8. *Giongo, J. L., Lucas, F. S., Casarin, F., Heeb, P., & Brandelli, A. (2007). Keratinolytic proteases of Bacillus species isolated from the Amazon basin showing remarkable de-hairing activity. World Journal of Microbiology and Biotechnology, 23(3), 375-382.*
9. *Green, G. H. (1955). The recovery of wool from sheepskin pieces. Journal of Applied Chemistry, 5(7), 296-313.*
10. *Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., ... & Beiko, R. G. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology, 31(9), 814.*
11. *Liu, W. W., Wei, M. U., Zhu, B. Y., Du, Y. C., & Feng, L. I. U. (2008). Antagonistic activities of volatiles from four strains of Bacillus spp. and Paenibacillus spp. against soil-borne plant pathogens. Agricultural Sciences in China, 7(9), 1104-1114.*
12. *Maxwell, M. E., & Lennox, F. G. (1944). Bacteria responsible for the loosening of wool on sheepskins. Nature, 154(3899), 118.*
13. *Naffa, R., Edwards, P. J., & Norris, G. (2019). Isolation and characterization of collagen type I crosslink from skin: high-resolution NMR reveals diastereomers of hydroxylysinonorleucine crosslink. Amino acids, 1-11.*
14. *Naffa, R., Maidment, C., Ahn, M., Ingham, B., Hinkley, S., & Norris, G. (2019). Molecular and structural insights into skin collagen reveals several factors that influence its architecture. International journal of biological macromolecules, 128, 509-520*
15. *O'reilly, T., & Day, D. F. (1983). Effects of cultural conditions on protease production by Aeromonas hydrophila. Appl. Environ. Microbiol., 45(3), 1132-1135.*
16. *Pan, A., Walsh, A., Dearth, M., & Zhang, X. Q. (2017). Validating Target Compounds to Vehicle Interior Odor Complaints by Reconstituting Their Concentrations in Vehicles (No. 2017-01-0324). SAE Technical Paper.*
17. *Raman, N. M., Asokan, S., Sundari, N. S., & Ramasamy, S. (2018). Bioremediation of chromium (VI) by Stenotrophomonas maltophilia isolated from tannery effluent. International journal of environmental science and technology, 15(1), 207-216.*
18. *Ranjithkumar, A., Durga, J., Ramesh, R., Rose, C., & Muralidharan, C. (2017). Cleaner processing: a sulphide—free approach for depilation of skins. Environmental Science and Pollution Research, 24(1), 180-188.*
19. *Rao, R. R., Vimudha, M., Kamini, N. R., Gowthaman, M. K., Chandrasekran, B., & Saravanan, P. (2017). Alkaline protease production from Brevibacterium luteolum (MTCC 5982) under solid-state fermentation and its application for sulfide-free unhairing of cowhides. Applied biochemistry and biotechnology, 182(2), 511-528.*
20. *Schröttner, P., Rudolph, W. W., Damme, U., Lotz, C., Jacobs, E., & Gunzer, F. (2017). Wohlfahrtiimonas chitiniclastica: current insights into an emerging human pathogen. Epidemiology & Infection, 145(7), 1292-1303.*
21. *Thanikaivelan, P., Rao, J. R., Nair, B. U., & Ramasami, T. (2004). Progress and recent trends in biotechnological methods for leather processing. TRENDS in Biotechnology, 22(4), 181-188.*

22. *Wang, J., Ma, Z. Z., Chen, L., Sun, H. J., & Fan, W. K. (2018). Determination of the Emission of Volatile Organic Compounds from Leather Seats in Environmental Test Chamber. In Key Engineering Materials (Vol. 768, pp. 31-35). Trans Tech Publications.*
23. *Xu, B., Chen, X., & Xiong, J. (2018). Air quality inside motor vehicles' cabins: A review. Indoor and Built Environment, 27(4), 452-465.*
24. *Yousuf, J., Thajudeen, J., Rahiman, M., Krishnankutty, S., P. Alikunj, A., & A. Abdulla, M. H. (2017). Nitrogen fixing potential of various heterotrophic Bacillus strains from a tropical estuary and adjacent coastal regions. Journal of basic microbiology, 57(11), 922-932.*
25. *Yu, X., Torzewska, A., Zhang, X., Yin, Z., Drzewiecka, D., Cao, H., ... & Wang, L. (2017). Genetic diversity of the O antigens of Proteus species and the development of a suspension array for molecular serotyping. PloS one, 12(8), e0183267.*
26. *Zhang, Y., Mansel, B. W., Naffa, R., Cheong, S., Yao, Y., Holmes, G., ... & Prabakar, S. (2018). Revealing molecular level indicators of collagen stability: minimizing chrome usage in leather processing. ACS Sustainable Chemistry & Engineering, 6(5), 7096-7104.*